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Paclitaxel loaded hybrid magnetic-lipid nanoparticles as a novel approach for breast cancer therapy

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Abstract

Cancer is the third leading cause of morbidity worldwide, only surpassed by cardiovascular and infectious diseases. In addition, breast cancer is the most common cancer among women around the world. Despite increasing research in recent years, real progress impacting on translational medicine is scarce. One of the main problems is current treatments are harmful for non-cancer cells, hence a more targeted approach may impact the efficiency of therapeutics. Nanotechnology promises to make an outstanding contribution to targeted therapy by enabling site-specific release of chemotherapeutic agents and because of its high stability, high carrier capacity, and greater ability to administer both hydrophilic and hydrophobic substances by several routes.

Nanostructured lipid carriers (NLC) and superparamagnetic iron oxide nanoparticles (SPION) appear as hopeful partners in the administration of antineoplastic drugs, such as Paclitaxel (PAC). NLCs are biocompatible nanometric sphere-like meshes of lipids which have the ability to encapsulate drugs within its pores, travel through the blood stream undetected, slowly and controllably releasing the contained drug and delivering it to, and only to, the target location. A magnitude smaller, SPIONs are an agglomeration of iron oxides, usually in a sphere shape, very prone to modulation. They can be used in diagnostics, therapeutics and even both at the same time.

Herein, SPION-NLC hybrid nanoparticles were studied as possible carriers for PAC. The NLC formulation is composed by a spherical nanoparticles suspension with a low polydispersity index around 0,237, a mean size diameter of around 375 nm, a zeta potential superior to |30| mV and a high encapsulation efficiency of 77%. Despite showing higher zeta potential values when stored at room temperature, the nanoformulation proved to be stable for at least 2 months at both 4ºC and 25ºC. Breast cancer cells, namely MDA-MB-231 cells, were used to evaluate the cytotoxicity of the nanoformulations. Internalization of the nanoparticles by MDA-MB-231 cells was proved both by an uptake assay and scanning electron microscopy (SEM). As demonstrated by methylthiazolydiphenyl-tetratozium bromide (MTT) assay SPION-NLC-PAC were efficient in inducing cell death of MDA-MB-231. Furthermore, results obtained with flow cytometry regarding apoptosis suggested the nanoformulations could indeed be valuable in the breast cancer therapy.

In conclusion, this nanoformulation still shows a lot of potential for breast cancer therapy. Nevertheless, further investigation is required, more cell viability tests should be performed, a wider range of concentrations and incubations times should be studied and other breast cancer cell lines should also be evaluated.

Keywords: lipid nanoparticles, SPIONs, breast cancer, MDA-MB-231 cells, cell viability.

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Resumo

O cancro é a terceira causa de mortalidade em todo o mundo, apenas ultrapassada por doenças cardiovasculares e infecciosas. O cancro da mama é o cancro mais comum entre as mulheres de todo o mundo. Neste sentido, a investigação na área do cancro tem crescido e recebido cada vez mais investimentos e apoios. Contudo, avanços sérios e aplicáveis estão tão longe ser alcançados como há décadas atrás. Um dos grandes obstáculos nas terapias antitumorais actuais consiste no facto de células saudáveis serem também atacadas e danificadas. Com efeito, uma abordagem direccionada a apenas células cancerígenas parece ser promissora. A nanotecnologia tem contribuído significativamente na evolução das terapias direccionadas uma vez que permite a libertação dos agentes quimioterapêuticos num local específico e porque demonstram grande estabilidade, grande capacidade de carga e conferem oportunidade de administração de fármacos hidrofilicos e hidrofóbicos por diversas vias.

Os transportadores lipídicos nanoestruturados (TLN) e as nanopartículas superparamagnéticas de óxido de ferro (NSOF) surgiram como bons parceiros na administração de fármacos antineoplásicos. Os TLNs são esferas lipídicas de escala nanométrica biocompatíveis, com capacidade de encapsular fármacos, viajar pela corrente sanguínea indetectáveis, libertar o fármaco contido de forma lenta e controlada no local-alvo. Com um tamanho dez vezes menor, as NSOF são aglomerados de óxido de ferro, normalmente de forma esférica, muito facilmente modulados. Tem a capacidade de realizar uma multitude de funções, desde diagnóstico a terapia, e até mesmo ambos simultaneamente.

Neste projecto, nanopartículas híbridas de TLNs e NSOFs foram estudados como possíveis transportadores de paclitaxel (PAC). A nanoformulação é composta por uma suspensão de nanopartículas esféricas com baixo índice de polidispersão, cerca de 0,237. A média do diâmetro das partículas é aproximadamente 375 nm e o potencial zeta é superior em módulo a 30 mV. As nanopartículas apresentam também uma elevada eficiência de encapsulação do fármaco, cerca de 77%. Apesar das nanopartículas armazenadas a temperatura ambiente apresentarem valores de potencial zeta mais elevados, tanto as armazenadas a 4ºC como a 25ºC provaram manter-se estáveis por pelo menos 2 meses. Células de cancro da mama, especificamente células MDA-MB-231, foram utilizadas para testar a citotoxicidade das nanoformualções. A internalização das nanopartículas por parte das células MDA-MB-231 foi comprovada através de ensaios de uptake e por microscopia electrónica de varrimento (MEV). Nos ensaios com brometo de 3-(4,5-dimetiliazol-2- il)-2,5-difeniltetrazólio em células MDA-MB-231 verificou-se citotoxicidade por parte das nanopartículas de interesse. Da mesma forma, os ensaios de apoptose realizados por citometria de fluxo demonstraram que a nanoformulação sugerida poderá ser valiosa na terapia contra o cancro da mama.

Em suma, a nanoformulação desenvolvida apresenta ainda grande potencial para aplicação em terapia do cancro da mama. Porém, são necessários mais estudos, mais testes que avaliam viabilidade celular devem ser realizados e uma maior gama de concentrações e tempos de incubação devem ser testados. É igualmente importante avaliar outras linhas celulares de cancro da mama.

Palavras-chave: nanopartículas lipídicas, NSOFs, cancro da mama, células MDA-MB-231, viabilidade celular.

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List of Abbreviations

7-AAD	7-Aminoactinomycin D
ANOVA	Analysis of Variance
Bcl-2	B-Cell Lymphoma 2
BRCA	Breast Cancer Gene
CrEl	Cremophor EL
CT	Computed Tomography
DLS	Dynamic Light Scattering
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EDS	Energy Dispersive Spectroscopy
EDTA	Ethylenediaminetetraacetic Acid
EE	Encapsulation Efficiency
ELS	Electrophoretic Light Scattering
EPR	Enhanced Permeability and Retention
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
GRAS	Generally Recognised as Safe
HER2	Human Epidermal Growth Factor type 2 Receptor
HPH	High Pressure Homogenisation
MDR	Multidrug Resistance
MRI	Magnetic Resonance Imaging
MTT	Methylthiazolydiphenyl-tetratozium Bromide
NLC	Nanostructured Lipid Carriers
NP	Nanoparticle
PAC	Paclitaxel
PBS	Phosphate Buffered Saline
PDT	Photodynamic Therapy

PE	Phosphatidylethanolamine
PEG	Polyethylene Glycol
Pen-Strep	Penicillin-Streptomycin
PET	Positron Emission Tomography
PI	Propidium Iodide
PLA	Polylactic Acid
PLGA	Poly(lactic-co-glycolic acid)
PS	Phosphatidylserine
QD	Quantum Dot
SD	Standard Deviation
SEM	Scanning Electron Microscopy
SLN	Solid Lipid Nanoparticle
SPION	Superparamagnetic Iron Oxide Nanoparticle
TNF	Tumor Necrosis Factor
US	Ultrassonic
USA	United States of America

Chapter 1 - Introduction

1.1 Nanotechnology and Nanomedicine

In 1959, the renowned physicist and Nobel Prize-winner Richard Feynman envisioned the possibility to manipulate atoms and molecules using high precision instruments. Later in 1981, the term nanothecnology was first applied by the engineer Eric Drexler when describing in more detail Feynman's speech [1,2]. Nanotechnology represents the understanding and control of matter at dimensions between approximately 1 and 100 nanometers, where unique phenomena enable novel applications [1]. As the particle size diminishes the surface area to volume ratio increases, promoting changes in its physicochemical properties and environmental interactions reaching a scale at which quantum effects dominate properties of materials [3].

Nanotechnology has proven to benefit society in a wide variety of areas, it has revolutionize a number of technology and industry sectors, such as electronics, energy, textiles, military, and others [4]. However, the wide range of studies on nanotechnology focus mainly on nanomedicine, in the last 20 years over 14 000 studies were published in the field. Nanomedicine is the medical application of nanotechnology, presenting new and innovative interventions and treatments. The three main sectors of nanotechnology - nanomaterials, molecular nanotechnology and biotechnology - might be an imperative tool for future medicine with application in diagnostics to therapeutics [2].

The nanoscale effects and increased surface area have triggered an investigation of nanomaterials as promising tools for the advancement of diagnostic biosensors, drug and gene delivery, and biomedical imaging [5,6]. Nanodiagnostics can possibly work at a cellular and even sub-cellular level, assuming extreme importance in the continuous goal to identify a disease as early as possible. In fact, a diversified set of nanoparticles has exhibited a prominent potential for detecting disease markers, fragments of viruses, pre-cancerous cells, specific proteins, antibodies, and others [6].

Superparamagnetic iron oxide nanoparticles coated with a recognition molecule represent an useful improvement to the typical Magnetic Resonance Imaging (MRI) technique since it provides a localized contrast [7]. Optical detection is also possible when a fluorescent molecule is encapsulated or used in the functionalization of a nanoparticle [8]. Quantum dots have also been thoroughly studied due to their unique physiochemical properties, conferred by a quantum confinement effect [9]. These types of nanoparticles show a wide range of applications such as molecule tracking and fluorescent imaging [10,11]. Nanosensors are another important example of the application of nanotechnology in diagnostics. Sensors are able to detect events or changes in the environment and provide a corresponding output, consisting in

electrical or optical signals. In this case, a nanosensor recognizes a specific molecule or a variation in the environment and transduces a signal that can be a magnetic resonance change, a release of light, or an absorbance shift due to agglomeration [12–14].

Several nanomaterials are currently under intense investigation, including nanowires, quantum dots, and iron oxide, in order to develop nanosensors constructed to detect disease. In particular, functionalized nanowires have been able to detect disease indicators and cancer cells, and to measure blood chemistry. In addition a sensor using billions of carbon nanotubes has been developed by MIT and Harvard researchers. These nanotubes were functionalized with antibodies that attach to cancer cells and appear to be sensitive enough to indicate the entrapment of a single cancer cell [15].

Identically, nanotechnology has demonstrated great potential in the area of therapeutics being able to improve drug's solubility and stability, prolong drug half-lives in plasma, minimize target effects, and concentrate drugs at the desired target site. Consequently, the term nanotherapy emerged as the use of novel nanoscale agents capable of a therapeutic effect at specific types of cells or extracellular locations [16,17]. Mostly, nanoparticles are applied as delivery agents, acting as vehicles for drugs, light-emitting or heat-releasing molecules, that can reach the targeted site in such a way that allows a directed treatment with minimal damage to bystander cells. Another major advantage for the application of nanotechnology in therapy is the possibility for controlled release of therapeutic compounds. Substantial investigation has been done in order to incorporate therapeutic agents into biocompatible nanodevices, such as liposomes, micellar systems, polymer nanoparticles, inorganic nanoparticles, nanotubes, and dendrimers [16,17].

Moreover, nanoparticles can also be engineered to stimulate body's innate repair mechanisms. Artificial activation and control of adult stem cells are an important step in this area of investigation. Magnetic nanoparticles and enzyme-sensitive nanoparticle-coatings that target brain tumors, polymeric nanoparticles coated with a red blood cell membrane (nanosponges) which act as decoys and intercept toxins in the blood stream, smart nanoparticles probes for intracellular drug delivery and gene expressing imaging, quantum dots that detect and quantify human breast cancer biomarkers, carbon nanotubes which can convert light into tightly focused sound waves, among others are just a few of the advances already achieved in nanotherapy [18,19].

Nanotheranostics emerged in recent years as the simultaneous implementation of nanotechnology for diagnosis and therapy. The recent advances in the area of nanotechnology has enabled a new generation of different types of nanomaterials to be useful to nanotheranostics applications with the intention to diagnose and treat the disease at its earliest stage, enhancing the prospective of cure. By combining both therapeutic and diagnostic functions in one delivery formulation, nanotheranostic agents enable disease diagnosis, therapy and real-time monitoring of treatment progress and efficacy, with only one pharmaceutical agent. High variability and

modification possibilities makes nanoparticles greatly desired for this approach [10,20–22]. Monitorization of biodistribution, drug release kinetics and therapeutic efficacy can be achieved by non-invasive imaging methods, such as magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET) and ultrasonic (US) imaging techniques. In combination with therapeutic strategies namely chemotherapy, gene therapy, photodynamic therapy (PDT), and photodermal therapy, nanotheranostics approaches can be acomplished [23].

Nanotherapy is widely studied with respect to the treatment for cancer, a disease with no predicted cure in the past. In fact, more than 80% of USA Food and Drug Administration approved and investigated drug nanocarriers are intended for cancer treatment [24].

1.1.1 Nanomedicine in Cancer

The human body is made up of trillion of living cells, that grow, divide and eventually die, being replaced by new cells. This is a process that is tightly regulated by their DNA machinery. However, when this orderly process fails, cells become abnormal, they persist in environments prone to cell death and new cells are formed when they are not needed. These cells can grow and divide uncontrollably and may develop the potential to invade or spread to other parts of the body. In this case, these cells are considered cancerous cells [25,26]. In fact, cancer is a genetic disease since it is caused by changes in genes that control several cell functions. These changes can be inherited or acquired during the individual's lifetime, they can be the result of errors in cell division or DNA damages caused by exposure to different environmental factors [25,27].

Remarkable progress has been accomplished towards the understanding of hallmarks of cancer progression and treatment. However, cancer continues to be the leading cause of death of the twenty-first century[28]. Basic biological processes that have an important role in the development of cancer have been extensively studied, researchers showed disturbances in growth factor binding, signal transduction, gene transcription control, cell-cycle checkpoints, apoptosis, and angiogenesis. New discoveries in cancer biology prompted the search for anticancer drugs, which lead to the design and development of a record number of novel compounds, from which some are currently being used in cancer therapy. Furthermore, the new therapeutic approaches are based on the rectification of the damage caused to the genes, by stopping the blood supply to the tumor or by destroying cancer cells. Despite all this new information and compounds there are still limitations to this kind of therapy. For instance, pharmacological active cancer drugs reach the tumor site with poor specificity and dose-limiting toxicity [29].

Nanotechnology is a rapidly growing field and promises to make an outstanding contribution to cancer treatment strategies by enabling site-specific release of chemotherapeutic agents, based on their physiochemical characteristics and

biological attributes [30]. Furthermore, nanoparticle formulations show high stability, elevated carrier capacity, and greater ability to administer both hydrophilic and hydrophobic substances through several routes [31]. Advances in synthetic chemistry allowed the development of different biological nanomaterials that can be used for numerous biological therapies, including drug delivery, cancer diagnosis, treatment and imaging [32].

Nanoparticle systems can possibly revolutionize cancer therapy, mainly due to their ability to reduce the tumor or related events without damaging healthy tissues. Moreover, nanoparticles offer higher efficacy, lesser side effects, site specificity, efficient delivery, and overcome multidrug resistance (MDR) [33]. Nanoparticle-based drug delivery systems such as liposomes, dendrimers, diamondoids, quantum dots (QD), viral nanoparticles, and carbon nanotubes have shown encouraging results in cancer therapy and drug delivery [34].

Table 1 - Nanoparticles for cancer therapy approved or in clinical trials.

Name	Nanocarrier	Formulation	Indication	Status	Ref.
CYT-6091	Inorganic nanoparticle	TNFα-PEG-gold	Solid tumors	Phase I	35
Doxil	Liposome	Liposomal doxorubicin	Ovarian, Breast Cancer	Approved 1995	36
NKTR-102	Micelle	PEG-micelle Irinotecan	Colorectal, Breast Cancer	Phase III	37
Abraxane	Protein nanoparticle	Paclitaxel-albumin	Metastic Breast Cancer	Approved 2005	38
Genexol-PM	Polymeric micelle	Miceller Paclitaxel	Breast, Lung, Pancreatic Cancer	Phase II-IV	39
Xyotax	Polymer-drug conjugate	Paclitaxel-poly-L-glutamic acid	Breast, Ovarian Cancer	Phase III	40
Oncaspar	Polymer-drug conjugate	PEG-L-asparaginase	Acute Lymphoblastic Leukemia	Approved 2006	41
BIND-014	Polymer nanoparticle	Docetaxel-PLGA/PLA-PEG with targeting ligand	Nonsmall Cell Lung Cancer, Prostate Cancer	Phase II	42
Zevalin	radio-immunoconjugate	anti-CD20 conjugated toytrium-90 or indium-111	Non-Hodgkin's Lymphoma	Approved 2002	43

The biggest advantage of using nanotechnology in cancer treatments is refined targeting. Targeting strategies can rely on ligand-mediated specific interaction between nanoparticles and cancer cell surface (active targeting) or by taking advantage of the abnormal tumor microenvironment (passive targeting). Ligand-targeted nanoparticles are expected to deliver cytotoxic agents selectively and specifically to tumor cells via receptor-mediated endocytosis, thereby enhancing intracellular drug accumulation. Antibodies, folate, growth factors, and cytokines are some of the several tumor-targeting ligands that can be used to facilitate the uptake of carriers into target cells [44]. Furthermore, monoclonal antibodies and antibody fragments are able to reduce immunogenicity and improve pharmacokinetics [45]. Passive targeting depends significantly of nanoparticles' properties, such as size, charge, surface nature, and circulation half-time [46]. Additionally, passive targeting to a tumor can be achieved due to the enhanced permeability and retention effect (EPR) [47]. EPR effect is the property by which molecules of certain sizes tend to accumulate in tumor tissue.

As previously depicted, nanotechnology-based approaches can also be applied to diagnosis, showing a great potential for improving the efficiency of early detection of precancerous and neoplastic lesions. Nanomaterials have long-term stability and allow *in vitro* and *in vivo* diagnosis with superior sensitivity than conventional imaging [48,49]. Additionally, combination of existing optical imaging technologies with sophisticated nanoparticle-based optical contrast agents for high-resolution in vivo cancer imaging has been successfully achieved. However, developing reliable early detection of tumors from blood-serum, other biological fluids, or any sample obtained through minimal or non-invasive procedures remains imperative [50].

Notably, there are several nanoparticle therapeutics directed to cancer treatment approved by the USA Food and Drug Administration (FDA) or in clinical trials. A small portion is summarized in **Table 1**.

1.1.2 Breast Cancer

There are over 200 different types of cancer each with its own biology, diagnosis and treatment [51]. Breast cancer is the most common cancer among women worldwide and in 2015 approximately 300 000 new cases of breast cancer were diagnosed in women in the USA [52,53]. In men, breast cancer incidence is rare, with only 2 350 new cases of breast cancer diagnosed in the USA during 2015 [52].

In order to better understand breast cancer it is important to have some basic knowledge about the normal structure of women's breasts (**Figure 1**). Female's breasts are made up mainly of lobules (also known as milk-producing glands), ducts (connective tubes responsible for the transportation of milk from the lobules to the nipple) and stroma (fat and connective tissue surrounding the ducts and lobules, blood vessels, and lymphatic vessels) [54,55]. Normal breast tissue goes through hormonal-dependent cycles of imbalance between proliferation and apoptosis during

menstrual periodicity, pregnancy and lactation, that when not properly regulated may result in the development of tumors. Breast cancer usually begins in the cells of the lobules or the ducts, however in some cases can also begin in the stromal tissue [52,54–56].

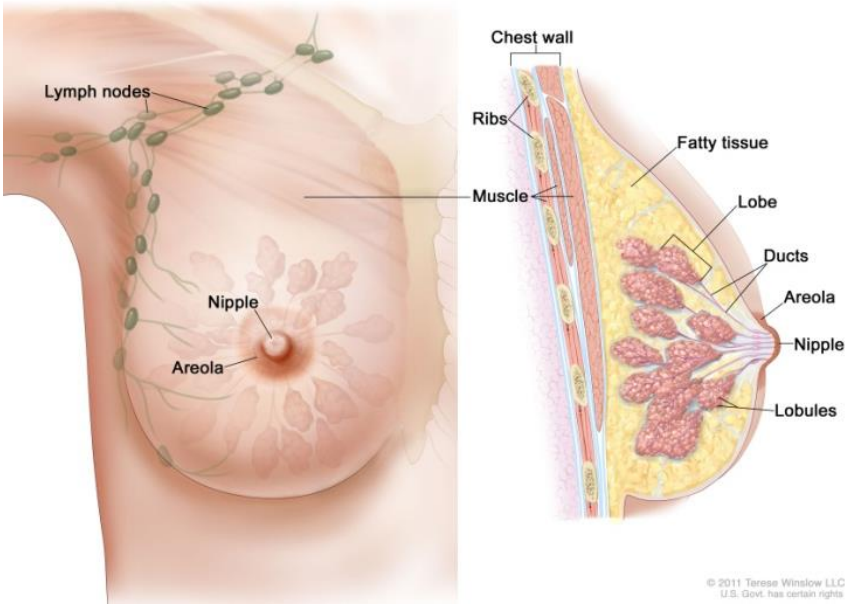

Figure 1 - Schematic representation of a female breast [58].

Human breast cancer is classified according to different systems: (1) the histological cell type, (2) the extent of invasion into surrounding tissues, (3) the size of the tumor, and (4) if it has spread to nearby lymph nodes. For instance, if the tumor has not developed the ability to spread outside the breast it is considered non-invasive (also known as carcinoma *in situ*), otherwise it is classified as invasive. Even though an invasive breast cancer has the ability to spread out it does not necessarily means it will. The cancer usually spreads out through the lymph nodes (small glands that filter foreign substances from the body) facilitating its access to the bloodstream. The more lymph nodes affected by breast cancer cells, the higher are the chances for the cancer to be found in other organs as well. However, not all women with breast cancer cells in their lymph nodes present signs of metastases, and cancer cells can be detected in other parts of the body in women with no cancer cells in the lymph nodes [52,54–56].

There are many risk factors for developing breast cancer, including being female, obesity, infrequent exercise, alcohol consumption, being under hormone replacement therapy during menopause, having a late pregnancy or none at all, age, among others [27,57]. Family history is also an important risk factor, about 5 to 10% of breast cancer cases are thought to be hereditary. The most common cause of hereditary breast cancer is an inherited mutation in the Breast Cancer Gene 1 and 2 (BRCA1 and BRCA2 genes). In normal cells, these genes help prevent cancer by making proteins that control and regulate their normal growth [57,58].

The first symptom noticed by women with breast cancer is normally a lump or an area of thickened tissue in the breast, but there are other symptoms such as, changes in size or shape of one or both breasts, abnormal discharge from the nipples, lumps or swelling on the armpits, dimpling on the skin of the breasts, a rash on or around the nipples, changes in the appearance of the nipples, pain in the breasts or armpits, and others [59]. However, even when none of the symptoms are presented, women should do routine breast cancer screenings (mammograms and breast ultrasounds). If the physician suspects of breast cancer diagnosis a biopsy should be performed in order to assess if the presence and type of breast cancer. There are four types of biopsy: excisional biopsy, where an entire lump of tissue is removed; incisional biopsy, in which only a part of a lump or a sample of tissue is collected; core biopsy, where a wide needle is used to remove tissue, and fine-needle aspiration biopsy, used to remove fluid from the lymph nodes or to drain a benign cyst without removing any tissue [60].

When choosing treatment the doctor must consider the cancer's stage and grade, the general health of the patient and whether the woman has been through menopause or is pregnant at the time [59,61]. To better assess future treatments a few tests are performed on the biopsy sample of the patient. In addition, these tests allow the physician to understand how quickly the cancer may grow, how likely it is to spread through the body and how likely it is to recur. Estrogen and progesterone receptor test measures the amount of these hormone receptors in cancer tissue, if there are more receptors than normal the cancer is described as estrogen and/or progesterone receptor positive. This type of breast cancer has a tendency to grow more quickly than others. Results from this test also provides valuable information about the viability of a treatment that blocks estrogen and/or progesterone. Human epidermal growth factor type 2 receptor (HER2) test is a laboratory test that measures the amount of *HER2* gene and HER2 protein in a tissue sample, if their levels are higher than normal the breast cancer is described as HER2 positive. This type of breast cancer tends to grow quickly and spread to other parts of the body, and is usually treated with drugs that target HER2 proteins, such as trastuzumab and pertuzumab. Although it is not routinely done, a multigene test can also be performed in order to study the activity of many genes at the same time in a tissue sample. These results may help predict cancer behavior. In conclusion, breast cancer may be described as Hormone Receptor Positive, Hormone Receptor Negative, HER2 Positive, HER2 Negative or Triple negative (estrogen receptor, progesterone receptor and HER2 negative) [62,63].

Treatment can be local or systemic. Local therapy intends to treat the tumor at the site, having no effect on the rest of the body, while systemic therapy consists in the oral or intravenous administration of drugs which can reach cells anywhere in the body. Surgery and radiotherapy are considered local therapies whereas chemotherapy, endocrine therapy and targeted therapy are described as systemic therapies [64,65].

1.1.2.1 Surgery

Surgery is usually the first treatment considered when breast cancer is diagnosed. Surgery has the main goal of removing the tumor and its margins for future analysis. After it has been removed and studied it is possible to tell if the tumor had clear margins, meaning that there are no cancer cells in the tissue surrounding the tumor [66]. There are different options of surgery depending on the size of the tumor, whether it has spread out to other parts of the body, the size of the breasts and patient's personal wishes and feelings. If the tumor and surrounding tissue may be excised without removing the entire breast, a lumpectomy will be performed (also known as breast-conserving surgery). On the other hand, if the whole breast has to be removed the doctor will recommend a mastectomy. A mastectomy can also be performed as a prophylactic measure in patients with high risk of developing breast cancer. Another prophylactic measure currently used is the removal of the ovaries since it will lower the amounts of estrogen in the body diminishing the risk for developing breast cancer [67,68].

Under certain circumstances, patients have the opportunity to decide between a mastectomy or a lumpectomy followed by radiation. The latter tends to be equally effective when the tumor is restricted to one site of the breast and is under four centimeters. It is also imperative that the tumor present clear margins. Possible side effects of any of these surgeries are pain, temporary swelling, tenderness, and hard scar tissue that forms in the surgical site. Bleeding and infection at the surgery site are also possible. During or after surgery women normally undergo reconstructive surgery to restore the breasts appearance. Nevertheless, many women decide not to have reconstruction or opt for a prosthesis instead [68–70].

1.1.2.2 Radiotherapy

Radiotherapy uses high-energy rays or particles that permanently damage the DNA of cancer cells, destroying them. However, nearby tissue also suffers temporary cell damage, causing side effects such as sore skin, fatigue and chest pain. In breast cancer, radiotherapy is usually administered after breast-conserving surgery to lower the chances of reappearance in the breast or in the nearby lymph nodes. Radiation is also recommended after a mastectomy when the tumor is larger than 5 cm or it has spread to the lymph nodes [71–73].

There are three main types of radiation, the most common is the external radiation (also known as external beam radiation) where a source of radiation is focused outside the body on the area affected by the cancer. The extent of radiation will depend on the type of surgery the patient was subjected to and whether any lymph nodes were affected. If the patient had a mastectomy where no lymph nodes had cancer, radiation will be targeted at the chest and the places where any drains exited the body. On the other hand, if a breast-conserving surgery was performed the whole breast will be getting radiation with an extra boost after the treatment is complete in

order to prevent recurrence. This boost is delivered by the same machine, though the beams are aimed directly at the area around the surgery site. If cancer is found in the lymph nodes under the arm, this area will also be subjected to radiation. Another way to deliver radiation is by seeds or pellets placed into the breast tissue next to the tumor, this is internal radiation (also known as brachytherapy). Internal radiations can be delivered using multiple small tubes or catheters that are inserted into the breast around the area where the tumor was removed. Each day, for short periods of time, radioactive pellets are inserted in the tubes. Once the treatment is complete the tubes and the pellets are removed. This technique is known as interstitial brachytherapy. Moreover, brachytherapy can be intracavitary, where a balloon-catheter device can be used to deliver radiation. This tube with a balloon is placed in the area where the tumor was and it is filled with fluid so it remains in place. Radioactive pellets are placed down through the tube and into the device for a short time. At the end the balloon-catheter device and pellets are removed. The latter is the most commonly used nowadays and it is considered a form of accelerated partial breast radiation. There is also the option of receiving radiation during surgery, known as intraoperative radiation or intrabeam radiotherapy. An applicator is positioned inside the breast after the tumor is removed, furthermore a radioactive source is placed inside this applicator for ten to thirty minutes, giving a high dose of radiation directly to the tissue around the cancer. This technique is fairly new and there is no consensus between doctors if this is a good alternative to external or internal radiotherapy. Besides the curative intent, radiotherapy may also be given as a palliative treatment to relieve symptoms and reduce the suffering cause by the cancer [71,72].

In some cases, radiotherapy can be combined with hyperthermia (also known as thermotherapy). An energy source, such as ultrasound or microwave, is used to heat cancer cells which can increase their sensitivity to radiation. This course of treatment is still under study and it is not available everywhere [74].

1.1.2.3 Chemotherapy

Chemotherapy involves the use of cancer-killing drugs that travel through the bloodstream to reach cancer cells and disrupt their growth. However, these drugs cannot distinguish between normal and cancer cells, having an effect in every actively growing and dividing cell. Consequently, these drugs will have a poisonous consequence on the body causing problems that include fatigue, hair loss, mouth sores, nausea and low blood cell counts. Furthermore, chemotherapy can affect cells from the bone marrow leading to an increased chance of infections and easier bruising and bleeding. Before any type of chemotherapy is chosen the doctor must consider the tumor type, tumor size, tumor grade, number of lymph nodes involved and its degree of involvement, and the risk for metastasis [75,76]. There are many cases where chemotherapy may be recommended, it can be administered as main treatment in cases where the cancer has come back or has spread outside the breast and underarm area, and it can be associated with surgery. When it is used in patients

with no evidence of cancer after surgery it is called adjuvant therapy since it is used to kill any cancer cells that may have been left behind or spread, but have gone unnoticed. Using this procedure the risk of breast cancer recurrence is highly reduced. When chemotherapy is administered before surgery it is called neoadjuvant therapy. Neoadjuvant chemotherapy has two main benefits, it can shrink the tumor allowing a less extensive surgery and it can give precious information on the response of the tumor to the treatment. For example, if the first set of drugs do not shrink the tumor, another combination of drugs will be needed. Chemotherapy may also be used as a palliative measure to relieve symptoms in cases where a cure is not possible [75–78].

In most cases a combination of drugs has been proved to be more effective, however there is no single combination that has shown to be the best. Furthermore, several clinical trials still compare today's most effective treatments to new ones in the hopes of improving breast cancer treatment. Anthracyclines and taxanes are the most common drugs used in early stage breast cancer, usually in combination with other drugs. On the other hand, when the disease is advanced it is often treated by one single drug, still some combinations like carboplatin or cisplatin plus gemcitabine are commonly used to treat advanced breast cancer [75,76].

1.1.2.4 Endocrine Therapy

Hormones are regulatory biochemical compounds produced by the body that control the growth and activity of normal cells. Female hormones, estrogen and progesterone, are mainly produced in the ovaries, but can also be produced in smaller amounts by the adrenal cortex. Estrogen is responsible for the regulation of the menstrual cycle and the reproductive system, and the development of secondary sex characteristics. Progesterone plays an important role in the menstrual cycle and during pregnancy, and promotes the development of the mammary gland. However, both of them can stimulate the growth of breast cancer cells. Hormone-sensitive breast cancer cells contain proteins known as hormone receptors. When hormones bind to these receptors they are activated, which in term will alter the expression of some specific genes, leading to the stimulation of cell growth. Therefore, endocrine treatment intends to lower the levels of female hormones or block their effects. However, treatment is more likely to succeed in hormone receptor-positive cancer cells, either estrogen receptor-positive or progesterone receptor-positive. Tamoxifen is a well-known estrogen receptor modulator developed over 30 years ago. It is a selective estrogen receptor modulator (SERM) since it acts as an anti-estrogen in breast cells, but it acts like an estrogen in other tissues (uterus, bones, etc.) [79,80].

Hormone therapy can be applied in different situations, before surgery to shrink large or locally advanced tumor in the breast, after surgery to lower the risk of reappearance, if the patient cannot have surgery to stop the growth and shrink the tumor, and if the cancer comes back or spreads to control or shrink a breast cancer

that has metastasize. Although endocrine therapy is a good option to treat breast cancer it can never get rid of it completely [79,80].

1.1.2.5 Targeted Therapy

The biggest problem with conventional cancer therapies is that they are not able to distinguish between normal and cancer cells, attacking both of them. To overcome this dilemma many studies have been carried out. The more knowledge about genetic alterations that lead to cancer the easier it becomes to develop new drugs that specifically target these changes. These targeted drugs present different mechanisms of action and fewer side effects than conventional chemotherapy drugs [81,82].

For instance, researchers have found that some breast cancer cells have extremely high levels of HER2, a growth-promoting protein, on their surface stimulating their growth and metastases. With this new information several drugs have already been developed to target this protein. Trastuzumab is one of the most largely used. It is a man-made version of a very specific immune system protein that attaches to HER2 and, as a result, slows the tumor growth. It may also stimulate the immune system increasing its efficiency in attacking cancer cells [81,82].

1.1.2.6 Nanotherapy

Within targeted therapies nanotherapy is definitely the most valuable. Nanotherapy by definition is the use of nanoscale strategies to produce therapeutic effects. Regarding breast cancer there are many nanoparticles under investigation but few are the ones that are approved by the USA FDA and European Medicines Agency. Nab-paclitaxel is a nanoparticle formulation approved by the FDA and already used in the treatment of breast cancer. Nab-paclitaxel is an alternative formulation where paclitaxel is bound to albumin nanoparticles and it proved to be better at reaching tumors than solvent-based Paclitaxel [83,84]. A non-pegylated form of liposomal encapsulated doxorubicin, known as Myocet, is also routinely used in the treatment of metastatic breast cancer. Myocet is currently approved in Europe and Canada, although it has not been approved by the FDA in the USA [85,86].

1.2 NANOFORMULATION

1.2.1 LIPID NANOPARTICLES

Lipid nanoparticles were first developed in the early 1990s. Today they are one of the most attractive nanoparticles for drug delivery, due to the combination of some characteristics, such as particle size, low toxicity, physical stability, controlled release

properties, high drug load and excellent tolerability [87]. Lipid nanoparticles are prepared as colloidal arrangements with a solid lipid matrix being held together by a surfactant. Its constituents are excipients recognised as safe and known to be biocompatible, therefore these nanoparticles are Generally Recognized as Safe (GRAS) [88].

Various techniques to produce lipid nanoparticles have been described. High Pressure Homogenisation (HPH) is a method that can be performed at both high (hot HPH) and low (cold HPH) temperatures. In the first the lipid and the drug are blended together and later combined with the surfactant at the same temperature. A hot pre-emulsion develops after high speed stirring of the solution, with the help of a temperature controlled high pressure homogeniser a nanoemulsion appears. Lipid nanoparticles are produced by the recrystallization of the nanoemulsion after it cools down to room temperature. Cold HPH synthetizes lipid nanoparticles after melting together both lipid and drug and subsequently placing the mixture in liquid nitrogen. Solid lipid microparticles appear and undergo high speed stirring in a cold aqueous surfactant solution, developing a pre-suspension. This pre-suspension is homogenised at room temperature and finally the lipid nanoparticles arise. Microemulsion is another technique where the solid lipids are melted and then dissolve the drug. A surfactant-cosurfactant solution at a temperature above the melting temperature of the solid lipids is later added with mild agitation in order to attain a microemulsion. Maintaining mild agitation, the micremulsion is dispersed in cold water developing ultrafine nanoemulsion droplets that immediately crystallize forming lipid nanoparticles. In solvent emulsification-evaporation the lipids are dissolved in a water-immiscible organic solvent and, under continuous stirring, emulsified in an aqueous phase containing surfactants. During the emulsification the organic solvent evaporates and the lipid precipitates producing lipid nanoparticles. To ensure thermodynamic equilibrium of liquids, water-miscible organic solvents saturated with water are employed in solvent diffusion. The transient oil-in-water emulsion is transferred into water under continuous stirring, leading to solidification of the dispersed phase and lipid nanoparticles synthesis due to diffusion of the organic solvent. Solvent injection is another common method in the production of lipid nanoparticles. Lipids are dissolved in a water-miscible solvent or mixture and later, with the help of an injection needle, the solution is rapidly injected into an aqueous surfactant solution. The double emulsion method is based on emulsion-evaporation method, and it is commonly known as water in oil in water (w/o/w). This technique uses a stabilizer during hydrophilic drug encapsulation in the internal water phase of a w/o/w double emulsion, avoiding drug partitioning to the external water phase during solvent evaporation. Another well-known technique is the high speed stirring and/or ultra-sonication where lipid microparticles are produced by spray congealing and are the used to produce lipid nanopellets by high speed stirring or sonication. Lastly, there is the emulsification-sonication method where the lipids are melted at temperatures slightly above their melting point. The drug dissolves in the lipids and an aqueous surfactant at the same temperature is later added to the solution. With the help of a high shear homogenization device the solution is homogeneously

dispersed and the obtained emulsion is then subjected to ultrasonication, leading to the synthesis of lipid nanoparticles [88–90].

There are mainly two types of lipid nanoparticles, Solid Lipid Nanoparticle (SLN) and Nanostructured Lipid Carriers (NLC) [90–92]. SLNs are prepared using solid lipids at room and body temperature and stabilized by surfactants. They are composed of 0.1-30% (w/w) solid lipid dispersed in an aqueous solution of 0.5-5% (w/w) surfactant [93]. These nanoparticles range from 40 to 1000 nm and can be used for target-specific controlled drug delivery by several administration routes. SLNs are able to incorporate both lipophilic and hydrophilic drugs because of their great water content [88,91]. Henceforth, they display a good physiochemical stability, protection and controlled release of the encapsulated drug, low toxicity, organic solvent free synthesis and an easy scale-up [88]. However due to their extremely ordered matrix and crystalline imperfections they show low encapsulation efficiency and little stability during storage, notably during storage polymorphic transitions may happen because of crystalline rearrangements leading to drug expulsion. Gelation of the dispersion and particle growth may also occur during storage [88,90,93,94].

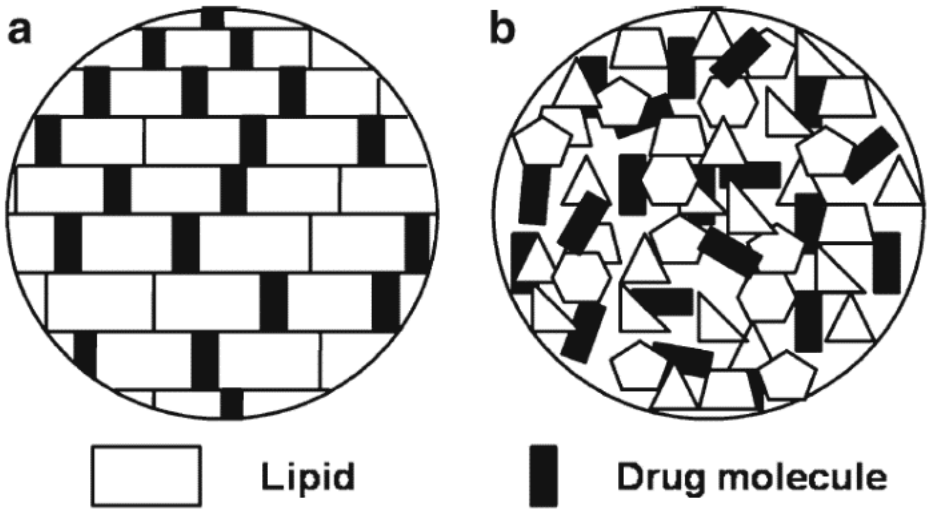

Figure 2 - Lipid crystalline matrix representation of SLN (a) and NLC (b) [88].

These limitations were surpassed by the development of another set of lipid nanoparticles, the NLCs. Therefore, a liquid lipid was added, allowing a less ordered lipid matrix and, consequently, higher encapsulation of the active compound (**Figure 2**) [95]. These nanoparticles show a targeted and controlled drug release and can also be easily adapted to large scale production [90,92,93,96]. As a result nanoresearchers have shown a keen oncological interest in NLCs, notably a wide variety of *in vitro* and animal models assays have been conducted successfully and reported in literature [97–104].

1.2.2 Magnetic Nanoparticles

Magnetism is a physical phenomenon mediated by magnetic fields. A magnetic field consists of imaginary lines of flux coming from the motion of electrically charged

particles, such as protons, electrons, and neutrons (despite being neutral, neutrons are basically smaller electrically charged particles) [105]. The magnetic field of an object can create a magnetic force on other objects with magnetic fields. Every material is influenced to some extent by a magnetic field, thereupon every material has an intrinsic property called magnetization, M. Magnetization is the vector sum of all its individual components' magnetic moments. When a material is placed in contact with an external magnetic field, H, its magnetization creates a larger induced magnetic field, B, in a phenomena called magnetic induction, where μ_0 represents the permeability of free space [106,107]:

$$\boldsymbol{B} = \boldsymbol{\mu_0}(\boldsymbol{M} + \boldsymbol{H})$$

Depending on the magnetic susceptibility of magnetic material, these can be classified in two categories, paramagnetism, when it is positive, and diamagnetism, when it is negative. According to Lenz's law, which states "If an induced current flows, its direction is always such that it will oppose the change which produced it", every material presents an innate diamagnetic property, even though it is very small in most cases [108,109]. Regardless, some materials display such a positive magnetic susceptibility that it will overpower its diamagnetic properties, in this case the material shows paramagnetism [110]. Another method of classification reckons on the relative ordering of the magnetic moments contained in a material. When there are no magnetic moments the material does not display diamagnetic properties. In a paramagnetic material each magnetic moment is randomly oriented due to thermal energy. However, when a decrease in temperature occurs, the magnetic interaction between the magnetic moments overcomes the thermal energy's influence, generating a characteristic ordered state. If the ordering is parallel and happens below the Curie-Weiss temperature we are in the presence of a ferromagnetic material. On the other hand, if the ordering antiparallel and occurs below Néel temperature the material shows antiferromagnetic properties. These critical transition temperatures are individual to each material's chemistry and magnetic characteristics. Ultimately, there is a variant of antiferromagnetism where each interacting pair of magnetic moments have different magnitudes [106,108–110].

When a magnetic material ranges from an atomic scale to a couple dozens of nanometers it starts behaving as a single giant magnetic domain, becoming similar to paramagnets [111]. Henceforth, magnetic nanoparticles mostly display superparamagnetism and are of keen interest to researchers. Usually, there are three functional parts in a magnetic nanoparticle, a magnetic core, a surface coating, and a functionalized outer coating. The magnetic core it is at the center of the nanoparticle and allows the manipulation of the nanoparticle in the presence of an external magnetic field [112,113]. Magnetic nanoparticles may be synthetize by physical vapour deposition, mechanical attritioning, and via chemical route [114]. There are a wide variety of applications for these types of nanoparticles, including magnetic fluids recording [115], catalysis [116], biotechnology/biomedicine [117], material sciences, photo catalysis [118], electrochemical and bioeletrochemical sensing [119], microwave absorption [120], MRI [121], medical diagnosis, data storage [122], environmental

remediation [123], among others. In biological applications, hyperthermia stands out, since it is a type of cancer treatment. Localized tissue is exposed to high temperatures, when it goes above 40ºC the natural enzymatic processes become more susceptible to the effect of radiation or chemotherapy while inducing apoptosis. Above approximately 45ºC human cells die of necrosis in a process known as thermoablation [124].

Furthermore, superparamagnetic nanoparticles have demonstrated to be a great advantage for therapeutics. In emphases, superparamagnetic iron oxide nanoparticles (SPIONs) show no agglomeration and no considerable cytotoxic effects, being a good option for cancer therapy [125–127].

1.2.3 PACLITAXEL

Taxanes are an important class of antitumor agents that act by stabilizing microtubules, leading to inhibition of cell proliferation. Paclitaxel (PAC) is a well-known taxane that was first identified as a crude extract of the Taxus brevifolia in 1966. It was later purified in 1969 and its structure was finally published in 1971. Approval from FDA marked a milestone in the management of breast cancer because it was the first agent to demonstrate efficacy after failure of combination therapy [128]. Paclitaxel binds to tubulin, the microtubules become locked in polymerized state, and thus the cells are restricted from G2 to M phase transition leading to ceased activity of essential cell processes, such as movement, chromosome segregation and cell division. Another effect later observed was it capability to inhibit the anti-apoptosis protein Bcl-2, and thereby induces apoptosis in cancer cells [129,130].

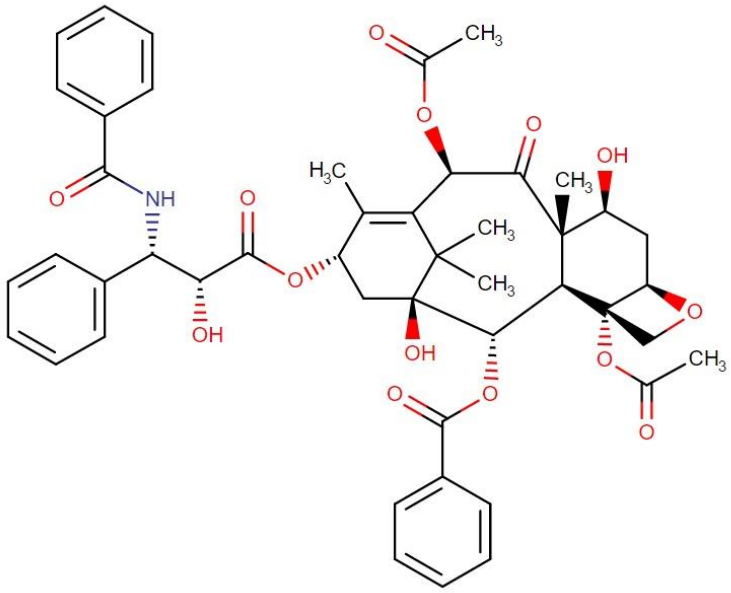

Figure 3 - Paclitaxel structural and chemical configuration [174].

Paclitaxel shows a tetracyclic heptadecane skeleton (**Figure 3**) which explains its low hydrophilicity (0,357 μg/mL) [131]. Notably, the clinical and experimental use of paclitaxel was limited in the beginning due to the poor solubility of the compound, and the demand for an excipient arose. Thus paclitaxel was primarily formulated in organic solvents, namely polyoxyethylated castor oil (Cremophor EL). Cremophor EL (CrEl) alters the pharmacokinetic profile of paclitaxel *in vivo* which displays an unpredictable non-linear plasma pharmacokinetics due to extensive binding to serum albumin, and consequent inactivity, broad tissue distribution, albeit incapable of passing blood-brain barrier, and a low half-life of 2.9±0.3 hours. Further, the effects are aggravated by the use of CrEl [132] [133]. Another combination attempting to solve paclitaxel's low solubility in aqueous media is Nab-paclitaxel, where paclitaxel is bound to albumin nanoparticles. This formulation proved to be better at reaching tumors than solvent-based Paclitaxel, it showed an improvement in the treatment response and a slower disease progression, while reducing systemic toxicity. In fact, Nab-paclitaxel is already used in the treatment of breast. Nonetheless, it presents a much larger cost, turning the better antitumor efficacy relatively marginal [83,84].

Henceforth, nanotechnology has become an intriguing strategy to overcome these drawbacks. The more obvious one is the physical and chemical protection it would provide by the entrapment of the pharmaceutical agent. It would also allow a higher injectable concentration and fully remove the excipient's presence and toxicity. Paclitaxel's pharmacokinetics would also be meliorated by the use of nanoparticle systems as a result of drug occlusion, a lack of epitopes detectable by the immune system, controlled release and possible surface modification, namely pegylation. A passive targeting can be achieved through the EPR effect, where particles with size less than circa 400 nanometers accrue in tumor tissue athwart damaged neo-angiogenic blood vessels. An active targeting can also partake with the help of molecules such as folic acid, transferrin and hyaluronic acid. Both types of targeting will promote a better biodistribution of paclitaxel. Indeed, nanotechnology has an immeasurable potential in versatility and functionality modulation. They can have a wide range of characteristics, from being positively, neutral or negatively charged, to being able to co-deliver multiple agents and/or present a theranostics combination. They can also be engineered to respond to environment stimuli, namely pH, heat, light or magnetic [134].

A large variety of nanoparticles have already been applied to the delivery of paclitaxel. Liposomes have shown an accentuated decrease in side effects and higher tumor inhibition of paclitaxel, however encapsulation efficiency is not the aspired since liposomes present an aqueous core which is not a good choice for hydrophobic drugs such as this. As reported by Cavalli et al (0.1% release in PBS during 2 hours [135]), Yegin et al (12.5-16.5% within 14 days [136]) and Lee at al (10% in 24 hours [137]), SLNs are a better option for such drugs since they show a solely lipd matrix, consequently presenting higher encapsulation efficacy and slower release profiles [135,137,138]. Although this may be true SLNs' matrix show some drawbacks, eliciting an increased interest in NLCs. Since it is a fairly new area of investigation

within lipid-based nanoparticles, there is little information about prosperous paclitaxel-loaded NLC formulations, making its research imperative [139–143].

1.3 AIM AND STRATEGY

In the last years, modern society has been facing a serious health problem with the rising of cancer incidence. In fact, cancer is still the leading cause of death of the twenty-first century. Furthermore, breast cancer is the most common among women and it shows a relatively high number of cases in men as well. Therefore, it is important to invest in the research of new and improved ways to attack breast cancer cells without damaging healthy cells. For this purpose nanotechnology may present great potential.

Paclitaxel is a drug commonly used in the treatment of breast cancer, however its hydrophobicity makes its administration a challenge. Once more, the key could be in applying nanotechnology concepts to the development of a nano-based delivery system for the carriage of the afore mentioned drug. The lipid matrix of NLCs presents a suitable environment for Pacitaxel, their surfactant-stabilized surface would hypothetically permit a great and long term stability in lipid media and they would offer occlusion and block Paclitaxel's destructive potential until the targeted site was reached. Magnetic nanoparticles, in particular SPIONs, may also offer advantages since their superparamagnetic behavior confers hyperthermic capabilities, which may have an additional therapeutic effect.

In this context, the present project aims to evaluate de cytotoxicity of SPION-loaded NLCs in breast cancer cells, namely MDA-MB-231 cells.

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Chapter 2 – Materials and Methods

2.1 MATERIALS

The lipids Compritol 888 ATO and Gelucire 43/01 pellets were a kind gift from Gattefossé (Gattefossé, France) and Miglyol 812 was purchased from Acofarma® (Terrassa, Spain). Tween® 80, Iron (II) Chloride tetra-hydrate 99%, Iron (III) Chloride hexa-hydrate 97% and Ammonium hydroxid 28-30%, propidium iodide were obtained from Sigma-Aldrich® (St. Louis, MO, USA). Acetonitrile was purchased from VWR International LLC (Radnor, PA, USA). Paclitaxel was bought from LC Laboratories (Woburn, MA, USA).Hanks' Balanced Salt Solution [-] CaCl2, [-] MgCl2 (HBSS), Dulbecco's Modified Eagle's Medium (DMEM), trypsin-EDTA (1x), Dulbecco's Phosphate Buffered Saline 10x pH 7.4 (PBS), Fetal Bovine Serum (FBS) and penicillin-streptomycin (Pen-Strep) antibiotics mixture were purchased from Gibco® by Life Technologies™ (Invitrogen Corporation, Paisley, UK).

All the weighting measurements were performed using a Kern ABT-120-SDM digital analytical balance (Kern & Sohn; Balingen, Germany). The microwave synthesis was carried out in a Discover® SP microwave system (CEM Corporation; Mathews, USA), automated by Explorer-12 software. Ethanol 70% was purchased from AGA (AGA-Álcool e Géneros Alimenares S.A., Lisboa, Portugal) Milli-Q water was purified by an Ultra-pure water system (Milli-Q, Sartorius, Arium® Cartige 1 and 2, Sartorius Stedin Biotech; Göttingen, Germany) by a reverse osmosis process.

2.2 METHODS

2.2.1 NLC SYNTHESIS

Lipid nanoparticles were prepared by a modified free organic-solvent hot emulsification/sonication method (**Figure 4**) [144]. Henceforth, 70% (w/w) of Gelucire® 43/01, 6% (w/w) of Compritol® 888 ATO, 10% (w/w) of Miglyol® 812, 12% (w/w) of Tween® 80 and 2% (w/w) of Paclitaxel were weighted and stored in a glass tube that tube was later placed in a water bath at 75ºC since the higher melting point within the lipids is of 70ºC. When all the lipids melted 15 mL of double distilled water, pre-heated at 75ºC were added, obtaining an opaque, white suspension, which was placed under the probe-sonicator at 104 W of potency during 10 minutes. The resulting white nanoemulsion was stored in a sealed glass vial and left to cool at room temperature.

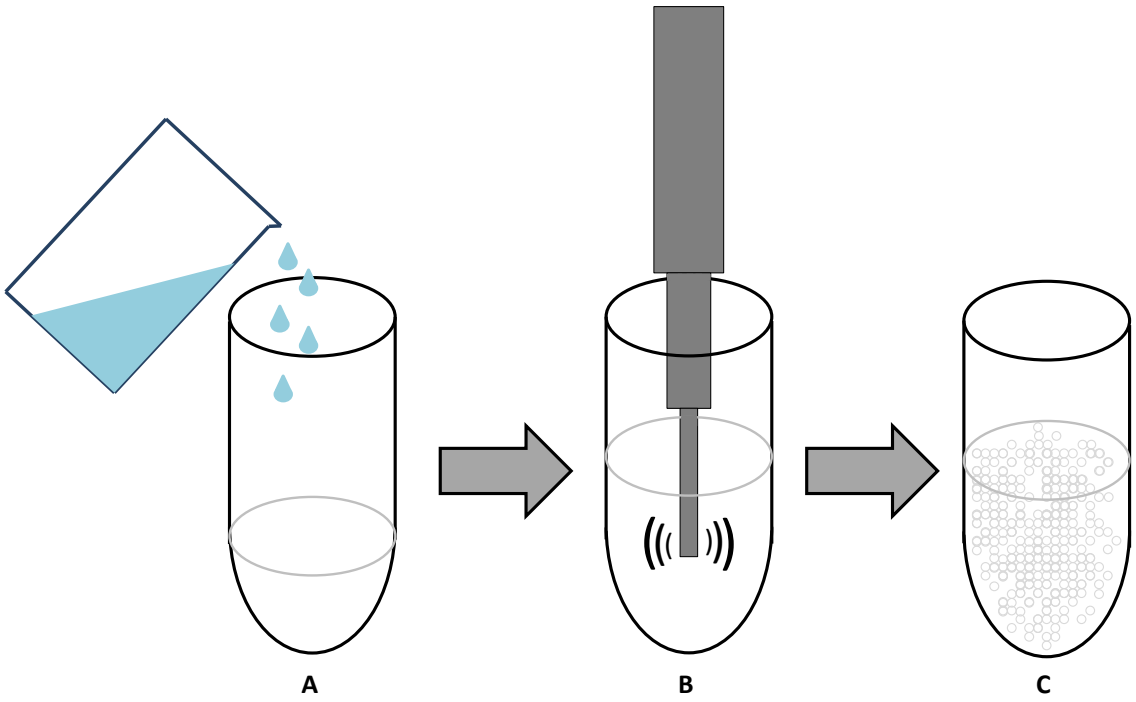

Figure 4 - Representative diagram of the NLC synthesis process by a standalone ultrasonication technique. Addition of water to melted lipid, surfactant and drug (A), sonication (B) and final product (B).

2.2.2 SPIONS SYNTHESIS

SPIONs were synthesized by a process described by Mahdavi et al[145], with a few modifications. Iron (II) Chloride tetra-hydrate ($FeCl_2 \cdot 4H_2O$) and Iron (III) Chloride hexa-hydrate ($FeCl_3 \cdot 6H_2O$), in a 1:1.8 molar ratio, were dissolved in 20 mL of double distilled water in a vessel under vigorous magnetic stirring. 3 mL of ammonium hydroxide was then rapidly added. After the precipitate settled, the vessel was placed inside the microwave system and the temperature set to 100ºC, under maximum stirring, during 10 minutes. After the elapsed time, pressured nitrogen gas was injected into the system to quickly lower the temperature until 70ºC to stop the reaction. Lastly, the black precipitate was collected with a strong permanent magnet, washed two times and suspended in 25 mL of double distilled water. The SPIONs were then stored in a 50 mL Falcon® at room temperature.

2.2.3 SPION-LOADED NLC SYNTHESIS

After the synthesis of NLC, as previously described in 2.2.1, 2 mL of SPIONs solution at room temperature were added (prepared as described in 2.2.2) obtaining an opaque, brown suspension, which was placed under the probe-sonicator at 104 W of potency during 10 minutes (**Figure 5**). The resulting brown nanoemulsion was stored in a sealed glass vial and left to cool at room temperature.

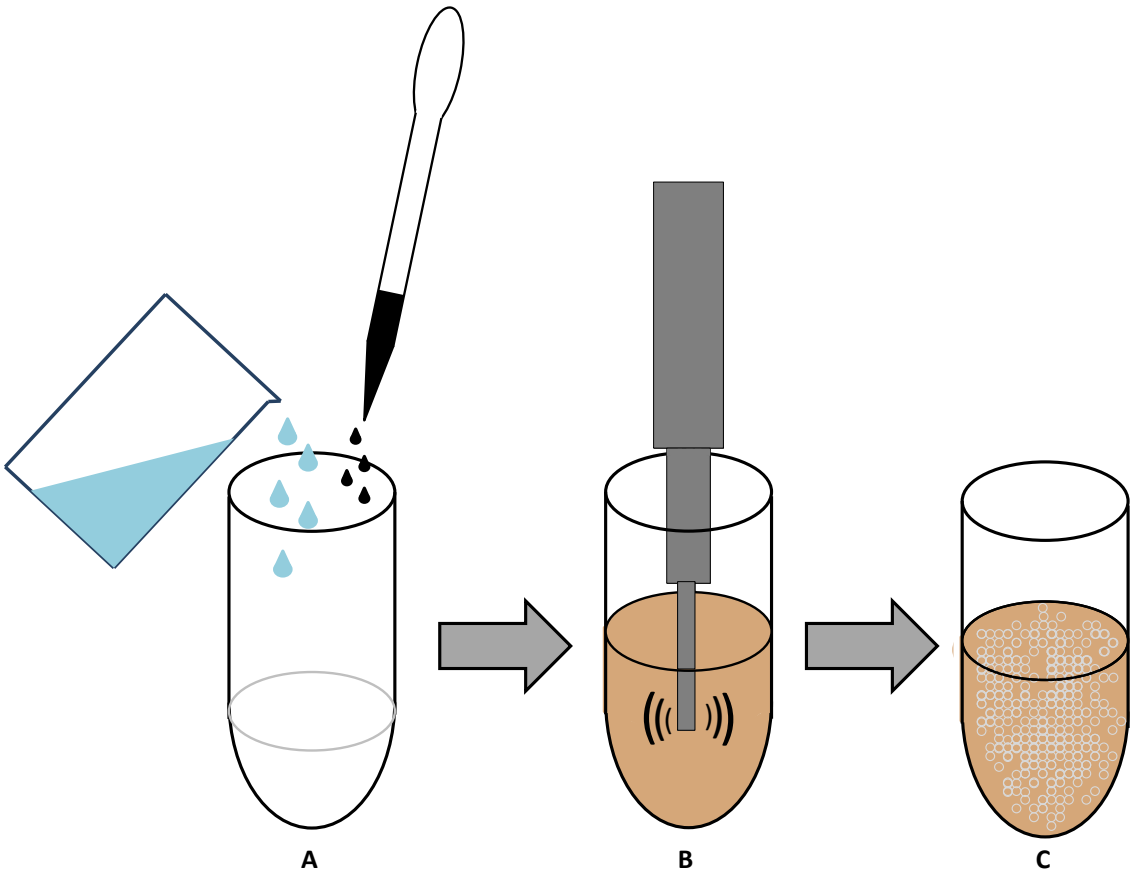

Figure 5 - Representative diagram of the SPION-loaded NLC synthesis process by an ultrasonication technique. Addition of water and SPIONS to melted lipid, surfactant and drug (A), sonication (B) and final product (C).

2.2.4 LYOPHILIZATION

After long periods of time, stored aqueous suspensions of NPs can form aggregates, and consequently become instable[146]. Therefore, the water is removed in order to improve the stability of the system. Lyophilizatin, a commonly used industrial process, removes water from a frozen sample by sublimination and desorption under vacuum. A cryoprotectant is often added to the formulations to protect the NPs against freezing and desiccation stresses[146].

The NLCs were lyophilized using a programmable freeze dryer (LyoQuest -85 laboratory freeze dryer, Telstar Grouo; Terrassa, Barcelona, Spain). Aerosil cryoprotectant was added to the NLCs suspensions before freezing at -80ºC in a GFL Upright Freezer 6481 (GFL Gesellschaft für Labortechnik mbH; Burgwedel, Germany). The aerosil was screened at the following concentration: 2% of total solid lipid mass for its cryoprotectant efficiency. The samples were lyophilized for 48 hours at -80ºC and pressure of 0.5 bar.

2.2.5 NLCs CHARACTERIZATION

2.2.5.1 Particle Size Measurements

Dynamic light scattering (DLS) is a common technique to determine particle size in colloidal suspensions[147]. The particles suspended in a liquid solvent present random movement, known as Brownian movement[147,148]. During the particle size measurement by DLS technique, the NPs suspension is exposed to a laser[149]. Due to the random motion of NPs, the direction and intensity of the incident light is changed in a process known as scattering. Thus, from these variations the diffusion coefficient of NPs can be measured. Considering that NPs are spherical particles, the hydrodynamic diameter of the particles can be calculated from their diffusion coefficients[149].

The produced NLCs were characterized by its mean hydrodynamic diameter and size distribution (polydispersiy index) in a Particle Size Analyser (Brookhaven Instruments Corporation; Software: Particle Sizing v.5 Brookhaven Instruments; Holtsville, NY, USA). The system was operating at a fixed light incidence angle of 90º, at 25ºC, with a dust cut-off set to 30 and the refractive index of the particles set to 1.329. Prior to measurements, NPs suspensions were diluted (0.5:100) in Milli-Q water. For each sample a total of 6 runs of 2 minutes each was performed.

2.2.5.2 Zeta Potential Measurements

Zeta potential is a physical property exhibited by particles in suspension that determines its stability. Therefore, the measurement of this potential can be useful to characterize the stability of NPs for an extended period. When particles are in contact with an aqueous solution, a rearrangement of the local free ions in the solution occurs, creating an unequal distribution of the solution ions in close proximity to the particle. Henceforth, an electrical double layer is formed, which is composed by a compact layer and a diffuse layer[150,151]. In the compact layer, the ions of the solvent are immobile due to the strong electrostatic attraction to the particle surface. On the other hand, in the diffuse layer the ions are mobile, since they have a weaker attraction to the particle surface[151] . The zeta potential is defined as the electrostatic potential at the boundary between the dispersion medium and the stationary layer of fluid attached to the dispersed particle[152]. Electrophoretic light scattering (ELS) is a common technique to measure the zeta potential. In this technique, two electrodes are inserted on the formulation. An incident light causes an electrophoretic movement of the particles in the electric field created by the electrodes[153]. The velocity of the particles motion to the electrode of the opposite charge is measured by the deviation of the laser light scattering spectrum, which allows the calculation of the zeta potential[154].

The zeta potential of all samples was determined using a Zeta Potential Analyser (ZetaPALS, Brookhaven Instruments Corporation; Software: PALS Zeta Potential

Analyser v.5 Brookhaven Instruments; Holtsville, NY, USA). The system was operating at 25ºC, with a fixed light incidence angle of 90º. The Smoluchowski mathematical model was used to perform the analysis. Prior to measurements, all samples were diluted (0.5:100) in Milli-Q water. For each sample a total of 10 runs, each one with 10 cycles, was performed.

2.2.6 PACLITAXEL ENCAPSULATION EFFICIENCY

2.2.6.1 Indirect Method – Sodium Salicylate

In this method, the encapsulation efficiency was determined by calculating the difference between the total amount of paclitaxel used to prepare the formulation and the amount of free paclitaxel remaining in the aqueous phase [155]. Formulations were diluted (1:100) in a solution of sodium salicylate (0,08 mg/mL) and transferred into Amicon® Ultra-4 Centrifugal Filter Devices, ultracel® - 50k (50000 NMWL) (MERK Milipore, Ltd; Cork, Ireland). Centrifugation was performed using an Allegra® X-15R centrifuge (Beckman Coulter; Pasadena, CA, USA) with a spin at 3500 rpm for 30 minutes or until complete separation between NLCs retained in the filter and the aqueous phase corresponding to the supernatant. The unentrapped paclitaxel was present in the supernatant. The EE was calculated as follows:

$$\text{EE}\ (\%) = \frac{\text{Total paclitaxel amount} - \text{Unentrapped paclitaxel}}{\text{Total pacltiaxel amount}} \times 100$$

A calibration curve of paclitaxel dissolved in sodium salicylate was obtained in order to correlate absorbance with concentration.

2.2.6.2 Direct Method – Acetonitrile

In the indirect method the encapsulation efficiency of paclitaxel was determined from the measurement of the amount of paclitaxel encapsulated in the NPs. Formulations were diluted (1:100) in Milli-Q water and transferred into Amicon® Ultra-4 Centrifugal Filter Devices, ultracel® - 50k (50000 NMWL). Centrifugation was performed using the Allegra® X-15R centrifuge with a spin at 3500 rpm for 30 minutes or until complete separation between NLCs retained in the filter and the aqueous phase corresponding to the supernatant. The filter unit was centrifuged in an inverted position at 4500 rpm during 10 minutes to isolate the pellet were NPs with the encapsulated paclitaxel were present. The pellet was then dissolved in 2 mL of acetonitrile. The entrapped paclitaxel was present in the solution of acetonitrile. Sometimes the solution of acetonitrile was centrifuged one more time before

analysed, the centrifugation was performed in a Milipore®, Low-binding Durapore PVDF membrane Ultrafree®-CL, at 4500 rpm for 10 minutes or until the lipids were deposited. The EE was calculated as follows:

$$\text{EE}\ (\%) = \frac{\text{Entrapped paclitaxel amount}}{\text{Total paclitaxel amount}} \times 100$$

A calibration curve of paclitaxel dissolved in acetonitrile was obtained in order to correlate absorbance with concentration.

2.2.6.3 Direct Method – Ethanol

In this method the encapsulation efficiency of paclitaxel was determined from the measurement of the amount of paclitaxel encapsulated in the NPs. Formulations were diluted (1:100) in Milli-Q water and transferred into Amicon® Ultra-4 Centrifugal Filter Devices, ultracel® - 50k (50000 NMWL). Centrifugation was performed using the Allegra® X-15R centrifuge with a spin at 3500 rpm for 30 minutes or until complete separation between NLCs retained in the filter and the aqueous phase corresponding to the supernatant. The filter unit was centrifuged in an inverted position at 4500 rpm during 10 minutes to isolate the pellet were NPs with the encapsulated paclitaxel were present. The pellet was then dissolved in 2 mL of ethanol and centrifuged at 4500 rpm for 30 minutes or until the lipids were deposited. The entrapped paclitaxel was present in the solution of ethanol. The EE was calculated as follows:

$$\text{EE}\ (\%) = \frac{\text{Entrapped paclitaxel amount}}{\text{Total paclitaxel amount}} \times 100$$

A calibration curve of paclitaxel dissolved in ethanol was obtained in order to correlate absorbance with concentration.

2.2.7 In Vitro Cell Viability Studies

MDA-MB-231 cells were cultured at 37ºC in a 5% CO_2 atmosphere (Unitherm CO_2 Incubator 3503 Uniequip; Planegg, Germany) in DMEM supplemented with 10% FBS and 1% Pen-Strep. When the cell culture reached a 70% to 80% confluence, was detached using a 0.25% trypsin-EDTA. Further, the cells were centrifuged using the Heraeus Multifuge X1R centrifuge (Thermo Fisher Scientific; Waltham, MA, USA) at

300 x*g* and re-suspended in fresh medium. Viable cells were counted using a Neubauer chamber (Improved Neubaur Bright-line, Boeco; Hamburg, Germany).

2.2.7.1 MTT Assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay is a colorimetric assay and it was the first homogeneous cell viability assay developed for a 96-well format,suitable for high throughput screening[156]. In this assay, viable cells convert MTT, a yellow tetrazole, into a purple colored formazan through mitochondrial activity. Dead cells lose the ability to reduce MTT into formazan, thus the quantity of formazan is directly proportional to the number of viable cells[157].

Therefore, the effect of the prepared NLCs on cell viability was evaluated using the MTT assay. The assay was performed for MDA-MB-231 cells. The collected cells were seeded in 96-well tissue culture test microplates (Tissue Culture OrPlates, surface treated flat bottom, Orange Scientific; Braine-l' Alleud, Belgium) at a density of $5x10^4$ cells/well in 100 µl of supplemented DMEM medium. All cells were incubated at 37ºC in a 5% CO_2 atmosphere until the cells presented 80 to 90% of confluence when observed using the Motic® AE2000 Binocular Inverted Microscope (Motic Electric Group Co., Ltd; Xiamen, Fujian, China). Then, the culture medium was discarded and 100 µl of NLCs formulations with different lipid concentrations (1 mg/mL, 5 mg/mL, 10 mg/mL, 15 mg/mL, 20 mg/mL and 25 mg/mL) were added to the cells, as well as a solution of Paclitaxel at different concentrations (0 µg/mL; 0,0117 µg/mL, 0,234 µg/mL; 0,352 µg/mL; 0,586 µg/mL; 0,117 µg/mL; 2,34 µg/mL; 3,52 µg/mL and 5,86 µg/mL). A positive control (only culture medium) was included to normalize and compare the results. The cells were incubated for 24 hours at 37ºC in a 5% CO_2 atmosphere. After the incubation time, the medium was discarded and 100 µl of MTT solution (5 mg/mL MTT in PBS stock solution diluted to a final concentration of 0.5 mg/mL in culture medium) was added to each well and incubated for 2h at 37ºC in a 5% CO_2 atmosphere, to allow the formation of formazan crystals. Then, the medium was rejected and 100 µl of DMSO were added to the wells, in order to dissolve the formazan crystals. The absorbance was measured at 590 and 630 nm using a microplate reader (BioTek Instruments; Winooski, VT, USA). The latter was used for background subtraction. Cell viability was determined according as follows:

$$\text{Cell Viability } (\%) = \frac{\text{Experimental absorbance}}{\text{Mean of absorbance of the positive control}} \times 100$$

2.2.7.2 Cell Viability by Flow Cytometry

Flow cytometry is a technique that simultaneously measures and then analyses multiple morphological characteristics of cells, as they flow in a fluid stream through a beam of light. Relative size, relative granularity or internal complexity, and relative fluorescent intensity are the properties measured with this technology. Furthermore, flow cytometry is a rapid and reliable method to quantify viable cells in a cell suspension[158].

MDA-MB-231 cells were seeded in 48-well plates at the density of 10^5 cells per well in 300 µl of supplemented DMEM and incubated at 37ºC in a 5% CO_2 atmosphere for 24 hours to allow cell attachment. Afterwards, the culture medium was discarded and 300 µl of NLCs formulations with different lipid concentrations (1 mg/mL, 5 mg/mL and 10 mg/mL) were added to the cells, as well as Paclitaxel solution at different concentrations (0.13 mg/mL, 0.07 mg/mL and 0.013 mg/mL) and SPIONs solution in a range of concentrations (0,5 mg/mL, 0,2 mg/mL and 0,05 mg/mL) A positive control (culture medium) was included to normalize and compare the results. The cells were incubated for 24 hours, 48 hours or 72 hours at 37ºC in a 5% CO_2 atmosphere. After incubation time, the medium was discarded and the cells were detached using a 0.25% trypsin-EDTA. Further, the cells were centrifuged using the Heraeus Multifuge X1R centrifuge (Thermo Fisher Scientific; Waltham, MA, USA) at 300 *xg*. Then, from the FITC Annexin V Apoptosis Detection Kit (BioLegend®; San Diego, CA, USA), 40 µL of Annexin V Biding Buffer, 2.5 µl of FITC Annexin V and 5 µl of 7-AAD Viability Staining Solution were added. After an incubation of 15 min at room temperature, 200 µl of Annexin V Biding Buffer were added and the samples were analysed by flow cytometry using the BD Accuri C6 (Biosciences; Belgium). For each sample a minimum of 20,000 events were recorded and the cells in culture medium were used as a control.

2.2.8 *IN* VITRO CELL UPTAKE

Flow cytometry is also a useful technique to assess the NLC uptake by MDA-MB-231 cells. Cells were seeded in 24-well plates at the density of $5x10^5$ cells per well in 400 µL of supplemented DMEM and incubated at 37ºC in a 5% CO_2 atmosphere for 24 hours to allow cell attachment. Afterwards, the culture medium was discarded and 400 µl of SPIONs-loaded NLC formulation at a 10mg/mL lipid concentration and of SPIONs solution at a concentration of 0.5 mg/mL. A negative control (culture medium) was included to normalize and compare the results. The cells were incubated for 24 hours, 6 hours, 3 hours, 1 hour or 30 min at 37ºC in a 5% CO_2 atmosphere. After incubation time, the medium was discarded and the cells were recovered by 0.25% trypsin-EDTA. Then, 5 µl of Propidium Iodide (PI) was added to excluded non-viable cells, and the samples were analysed by flow cytometry using the BD Accuri C6 (Biosciences; Belgium) recording the side scattering of each sample. For each sample a minimum of 20,000 events were recorded and the cells in culture medium were used as a control.

2.2.9 Scanning Electron Microscopy (SEM)

During scanning electron microscopy (SEM) a beam of high-energy electrons generates different signals at the surface of solid specimens. These signals reveal information about the sample, such as external morphology, chemical composition, and crystalline structure. Data is usually collected over a selected area and a 2-dimensional image is generated[159].

Hence, the morphology of the cells treated with NLCs was evaluated by SEM using high resolution scanning electron microscope with X-Ray microanalysis at the Materials Centre of the University of Porto, Portugal (CEMUP). MDA-MB-231 cells were seeded in 24-well plates at the density of $5x10^5$ cells per well in 400 µL of supplemented DMEM and incubated at 37ºC in a 5% CO_2 atmosphere for 24 hours to allow cell attachment. Afterwards, the culture medium was discarded and 400 µl of SPIONs-loaded NLC formulation at a 10mg/mL lipid concentration and of SPIONs solution at a concentration of 0.5 mg/mL. A positive control (culture medium) was included to compare the results. The cells were incubated for 24 hours at 37ºC in a 5% CO_2 atmosphere. After incubation time, the cells were washed and recovered after trypsinization. Further, the cells were attached into a microscope slide by centrifugation and analysed by SEM.

2.2.10 Statistical Analysis

Statistical analysis was performed using GraphPad Prism Software (v7 for Windows; GraphPad Software Inc, San Diego, CA, USA). The measurements were repeated at least 3 times and data expressed as mean ± SD. Data was analysed using two-way analysis of variance (ANOVA). A P value of <0.05 was considered statistically significant.

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Chapter 3 – Results and Discussion

3.1 NPs Characterization

The NLC formulation prepared by a modified free organic-solvent emulsification/sonication method[144] presented a white and milky appearance. The liquid suspension had a low viscosity and PAC deposits were not visible. **Figure 6A** shows the formulation which appeared stable by visual observation.

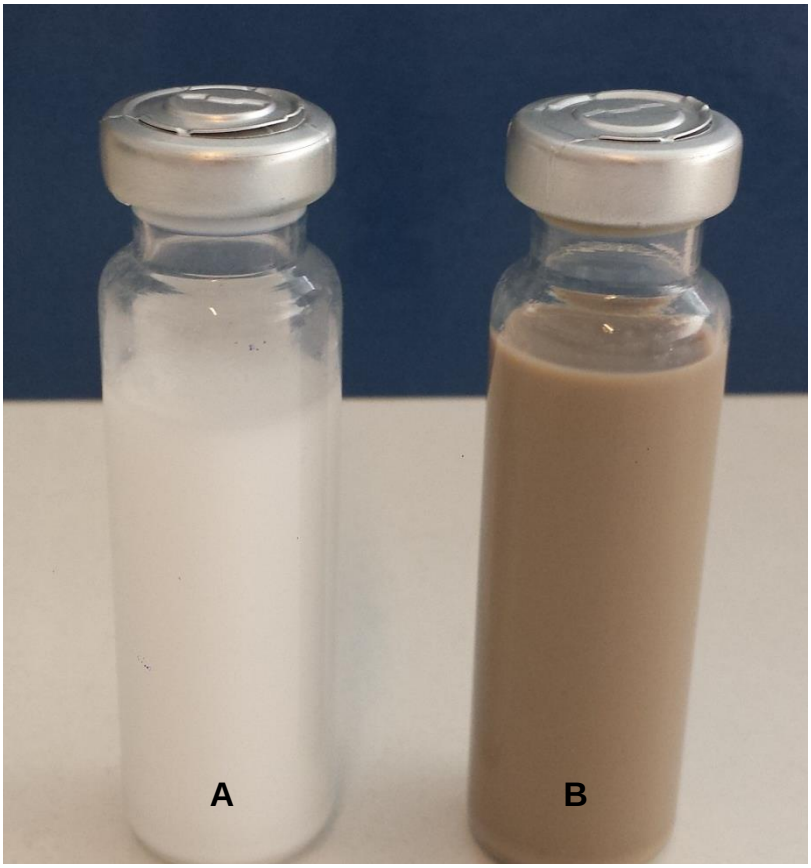

Figure 6- Lipid nanoparticles synthesized by emulsificaion/sonication: NLCs placebo (A) and SPION-NLC-PAC (B).

SPIONs solution synthesized by an adapted process described by Mahdavi et al[145] displayed a black tint and presented no signs of aggregation or precipitation, remaining stable in the solution (**Figure 7A**). To assure magnetic character a small permanent magnet was put into contact with the magnetic nanoparticles during a 5 second timeframe. Before the magnet was applied to the vial the solution is a completely dispersed, opaque, black suspension. At the moment of magnet exposure the nanoparticles are immediately attracted to the magnet, resulting in a progressive purification of the aqueous medium. All the SPIONs concentrate near the magnet forming a magnetic slurry. A solution of SPIONs is once more obtained by simple vigorous agitation (**Figure 7**).

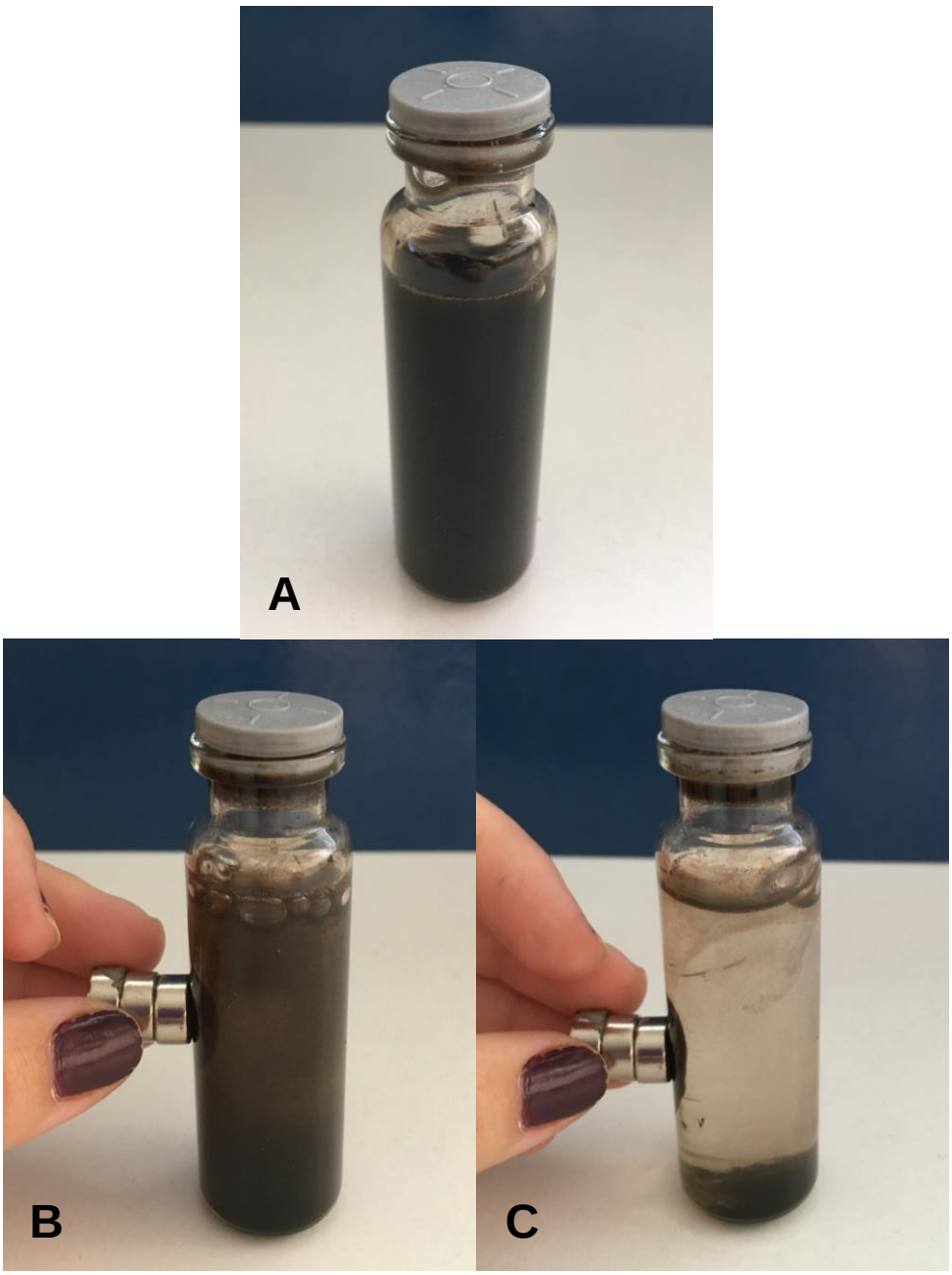

Figure 7 - SPION solution response to a small permanent magnet. SPION suspension state prior to magnetic exposure (A), 1 second after the magnet's placement (B), and 5 seconds of magnetic influence (C).

SPION-loaded NLCs were visually beige and milky as can be perceived in **Figure 6B**. The viscosity of the suspension was low and it appeared stable by visual observation. After synthesis, formulations were maintained in different conditions to evaluate the most suitable approach for long-term storage. Therefore, a batch was lyophilized at -80ºC during 48 hours to obtain a powder from the suspension. To compare the effect of the temperature in the formulations shelf-stability another non-lyophilized batch was divided into two vials, one was stored at room temperature and another one at 4ºC.

3.2 LYOPHILIZED NLCS

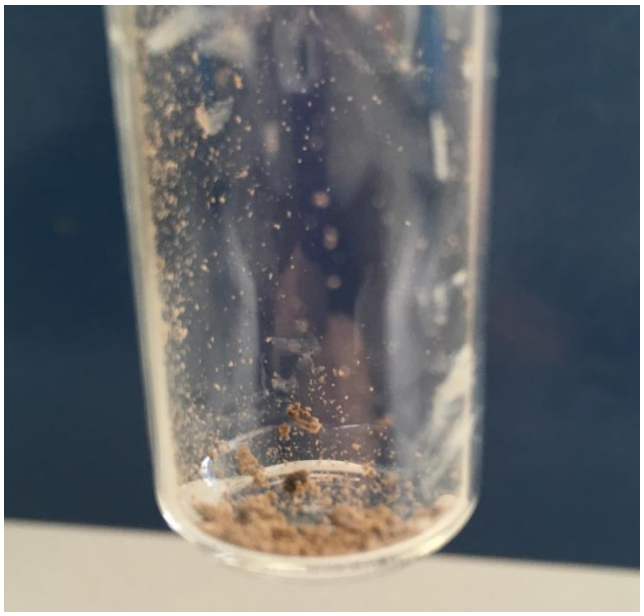

Figure 8 - Lyophilized NLCs.

The lyophilized NLCs were a light brown powder and were stored at 4ºC. The lyophilized NLCs showed some aggregation as it can be seen in **Figure 8**. Moreover, when it was necessary to resuspend the lyophilized NLCs in order to perform stability studies such process demonstrated to be ineffective. As previously reported, aggregation of nanoparticles depends on the lyophilisation process conditions and on the concentration of the cryoprotectant. Moreover, it may also be affected by the system of nanoparticles and the freeze drying conditions[146]. An optimization of the amount or even the type of cryoprotectant used should be performed to improve the process. Theoretically, a cryoprotectant, as the name suggests, can protect the nanoparticle from the mechanical stress of ice. However the selection of a proper cryoprotectant is not straightforward, some cryoprotectants, such as sucrose, have even been reported to destabilize the nanoparticles[146,160]. The freezing rate should also be taken into consideration since it is an important parameter in the first step of freeze drying[161].

According to the literature, the higher the concentration of cryoprotectant and the faster the freezing rate the lesser the aggregation[162], however the trend is reversed in several cases[160,163–165]. Thus, this complex process can only be developed by trial and error. Different concentrations and types of cryoprotectants should be tested, as well as various rates of freezing rates. Lyophilisation significantly improves long-term stability of nanoparticles, therefore optimizing this process is of upmost importance [146,166]. However, since it can be a time-consuming process, this methodology was for the time being, left behind. Consequently, a new approach where the stability of SPION-NLC hybrid suspension stored at different temperatures over time is evaluated was chosen.

3.3 STABILITY STUDIES

Stability studies were performed during the course of a month. Particle size, polydispersity and zeta potential were evaluated at weeks 0, 1, 2, 3 and 4 after the NPs synthesis and measured by DLS. The particle size of the SPION-NLC-PAC formulations stored at 4ºC and 25ºC was measured over time (**Figure 9)**. In both

cases, and with the exception of T0, the particle size remained constant through time revealing sizes between 350-390 nm. The mean size diameter is of around 375 nm.

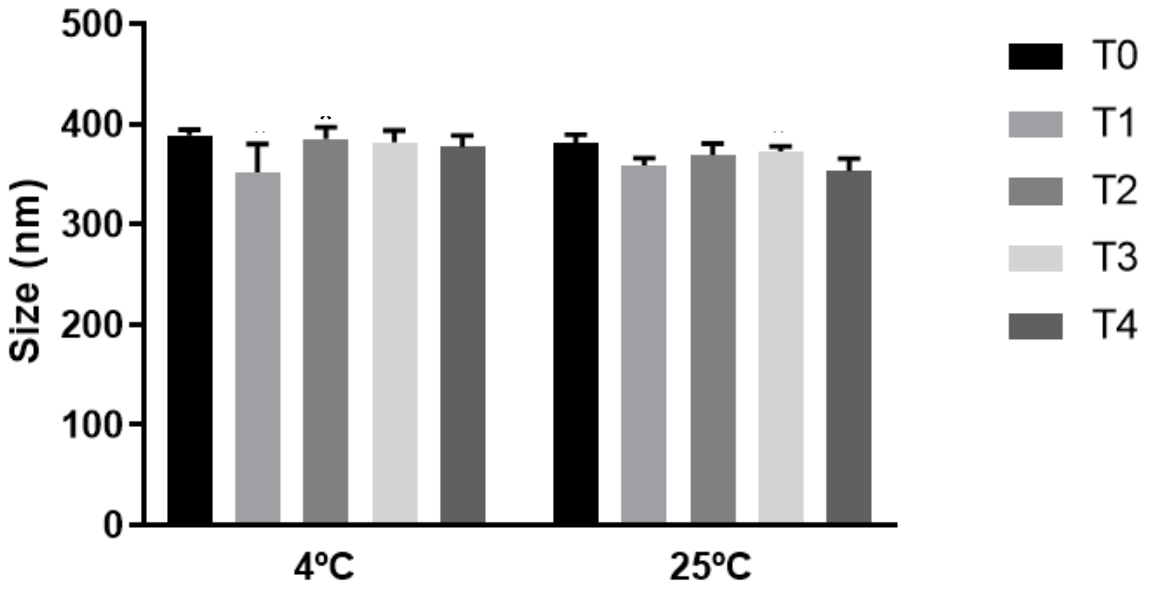

Figure 9 - Evaluation of the particle size of the formulations at 4ºC and 25ºC over time (0 (T0), 1 (T1), 2 (T2), 3 (T3) and 4 (T4) weeks). Bars represent the size (left Y axis). Values represent the mean ± SD of the readings.

Similarly, in both storage conditions, no significant changes in polydispersity were observed in comparison to week 0, except for week 4 (**Figure 10**). Suggesting that the polydispersity remains stable during this period of time. The nanoformulation shows a low polydispersity index of around 0,237.

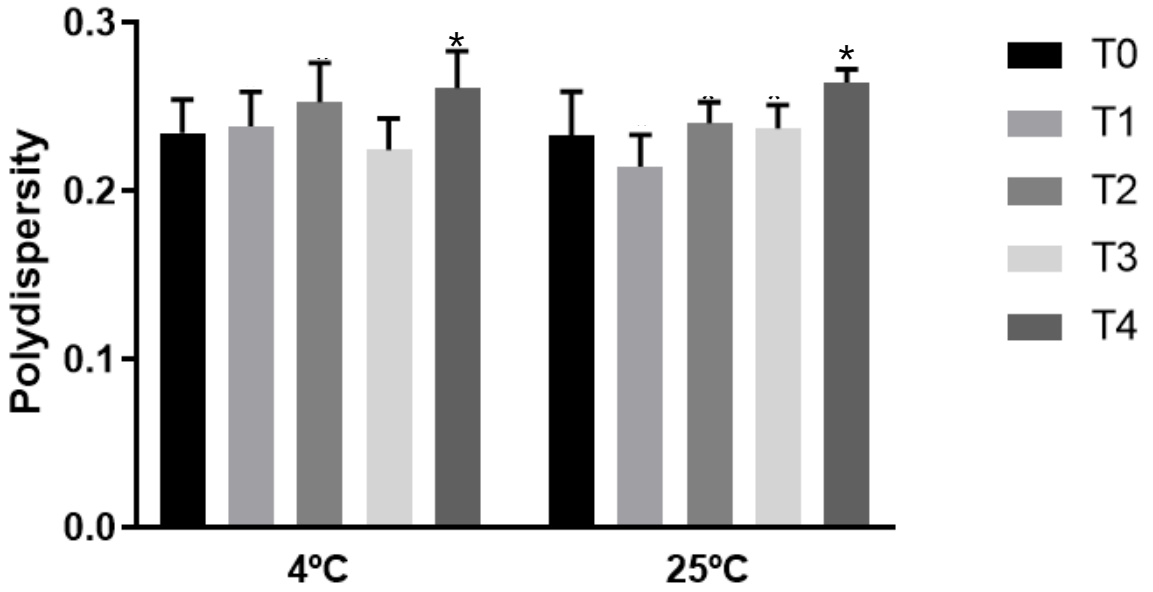

Figure 10 - Evaluation of the polydispersity index of the formulations at 4ºC and 25ºC over time (0 (T0), 1 (T1), 2 (T2), 3 (T3) and 4 (T4) weeks). Bars represent the polydispersity index (left Y axis). Values represent the mean ± SD of the readings. *P<0.05 relatively to the correspondent week 0.

The zeta potential is another important parameter to evaluate NPs stability. It characterizes surface charge, mutually influenced by the NPs composition and the medium where the NPs are dispersed[167]. Overall, if the absolute value of zeta potential is above 30 mV the dispersion is considered as stable owed to the electrostatic repulsion between the particles. However, if the zeta potential is between 5 and 15 mV there is limited flocculation and in cases where it is between 0 and 5 mV a maximum flocculation occurs[168]. Zeta potential values can be analysed in **Figure 11**. The SPION-NLC-PAC formulation stored at 4ºC showed zeta potential values between |25| and |38| mV, displaying a decrease in absolute value of zeta potential. In this case, it appears the nanoformulation is losing stability trough time. However, when stored at 25ºC the SPION-NLC-PAC formulation shows zeta potential values between |25| and |35| mV and appear to be maintaining their stability at an absolute value of zeta potential greater than 30 mV.

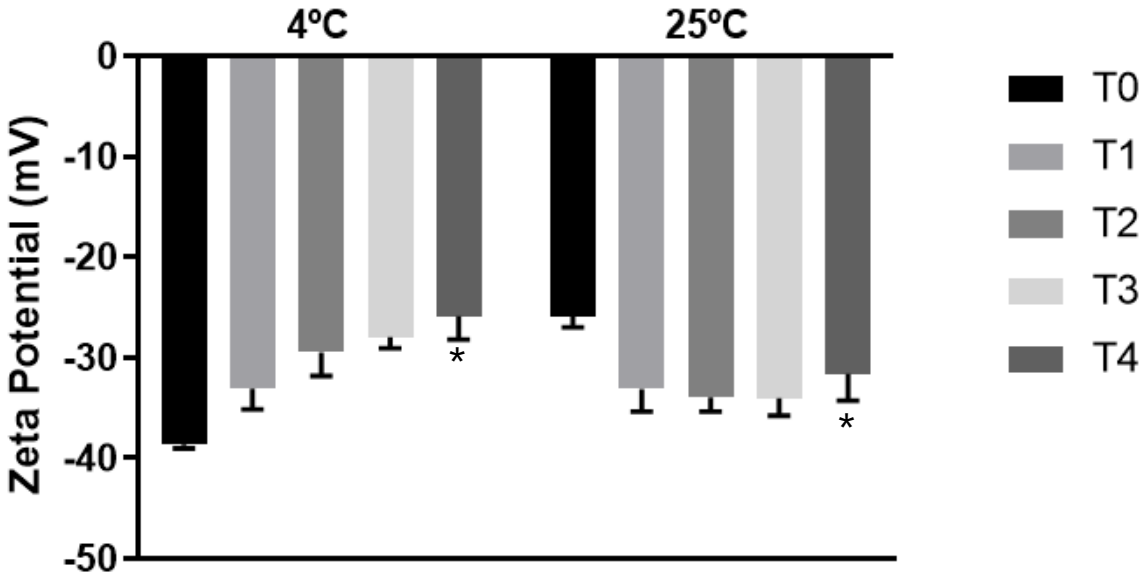

Figure 11 - Evaluation of the zeta potential of the formulations at 4ºC and 25ºC over time (0 (T0), 1 (T1), 2 (T2), 3 (T3) and 4 (T4) weeks). Bars represent the zeta potential (left Y axis). Values represent the mean ± SD of the readings. *P<0.05 relatively to the correspondent week 0.

Overall the results suggest that the SPION-NLC-PAC formulations present a higher stability when stored at room temperature.

3.4 Encapsulation Determination Optimization

Selecting an encapsulation protocol must take into consideration encapsulation efficiency, drug/lipid ratio, drug retention, ease of preparation, sterility, ease of scale-up, cost efficiency, as well as NP and drug stability. There are two main methods for the determination of encapsulation efficiency, the direct and indirect method. Whereas in the direct method the quantity of drug inside of the NP is directly measured, in the indirect method it is the amount of the non-encapsulated drug that is being quantified. In this report, both methods were tested.

In the indirect method the chosen solvent was sodium salicylate, due to its reported ability to dissolve the highly hydrophobic PAC[169]. Notwithstanding, a homogeneous solution could not be obtained, since several concentrations were tested and none presented the described solubility. Notably, this method was put aside and the direct approach became more appealing. Initially, acetonitrile was used as the solvent in the direct method since it promised high solubility for PAC[170]. However, after several attempts it proved to produce undesirable calibration, making it difficult to be a reliable approach for the EE determination.

Furthermore, ethanol, another solvent with high solubility for PAC[170,171], was applied to the direct method for the EE determination of the produced NLCs. At first, the method seemed to have its limitations since the SPION-NLC loading capacity for PAC obtained was higher than 100%. In light of these results, it was concluded that the lipids present in the nanoformulation should be interfering with the acquired absorbance value. To subtract this effect, placebo formulations, equally in every way except for the absence of PAC, underwent the same process and a mean of their absorbance values (Table 2) was then subtracted to the absorbance value obtained in the nanoformulation of interest.

Table 2 - Absorbance values obtained by direct method with ethanol as a solvent.

Samples	Absorbance (230 nm)	Mean
Placebo 1	0,229782	0,21558
Placebo 2	0,225157	
Placebo 3	0,204826	
Placebo 4	0,202554	
SPION-NLC-PAC	0,366621	

$$Final\ Absorbance\ value = 0{,}366621 - 0{,}21558 = 0{,}151041$$

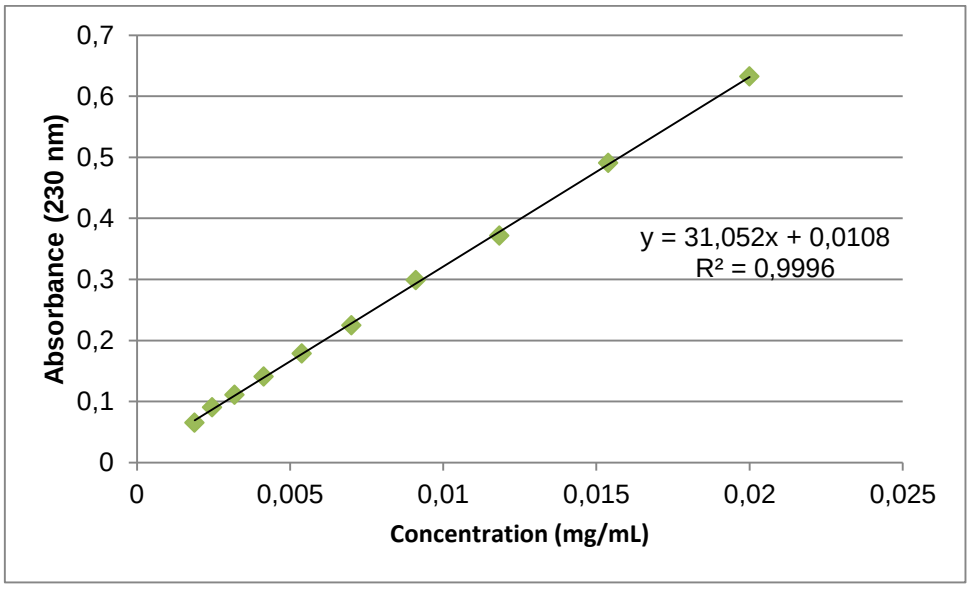

Figure 12- Calibration curve of PAC dissolved in ethanol.

Then with the calibration curve equation (**Figure 12**) where x represents the concentration and y the absorbance, a value for PAC's concentration inside the NLCs is obtained. Finally, the EE formula was applied and a value of 77% was obtained. Therefore, the SPION-NLC formulations are able to entrap 77% of the PAC added in their synthesis.

3.5 *In Vitro* Cell Viability Studies

In order to evaluate cytotoxicity of the developed NLCs on breast cancer cells, it is necessary to perform cell viability studies. MDA-MB-231 cells were selected to assess the effect of the NLCs on cell viability.

3.5.1 MTT Assay

A MTT assay was performed to assess the effect of developed NLCs on cellular viability of breast cancer cells, namely MDA-MB-231 cells. In this assay, viability of the cells incubated with SPION-NLC-PAC and free PAC at different concentrations in relation to the mean of positive control (incubated only with culture medium).

In **Figure 13**, decreased cell viability is clearly observed with the increase of SPION-NLC-PAC's concentration, after 24 hours treatment. Cell viability decreases to about 50% at 5 mg/mL of NLCs in lipid, indicating high cytotoxicity of SPION-NLC-PAC to MDA-MB-231 cells. At concentrations of 15, 20 and 25 mg/mL cell viability was close to zero.

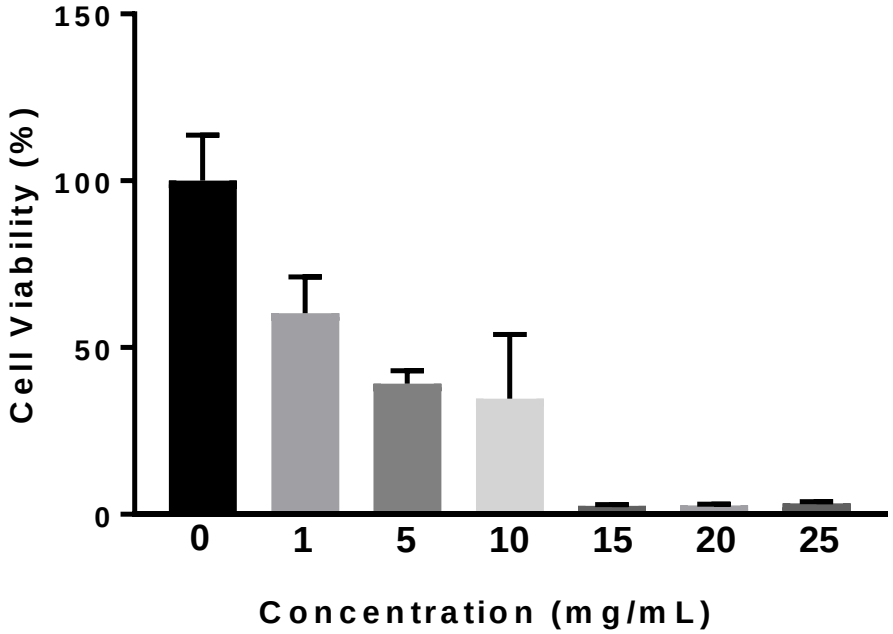

Figure 13 - MDA-MB-231 cell viability assessed by MTT assay as a function of the different concentrations of SPION-NLC-PAC tested (0 mg/mL, 1 mg/mL, 5 mg/mL, 10 mg/mL, 15 mg/mL, 20 mg/mL and 25 mg/mL). Values represent mean ± SD.

In regards to free PAC (**Figure 14**), a decline in cell viability can also be verified. The IC50 of PAC for MDA-MB-231 cells is 2,2 µM[172] (1,88 µg/mL), however this assay shows that at a concentration of 0,0853 µg/mL of free PAC 50% of cells have died. On the other hand, when comparing the IC50 value of PAC for MDA-MB-231 cells with the results obtained when these cells are treated with SPION-NLC-PAC, at a lipid concentration of 5 mg/mL (where 64,5 µg/mL of PAC is expected to be present) 50% of cell viability is lost, thus the effect of PAC on MDA-MB-231 cells is diminished when entrapped in SPION-NLC nanoparticles. This suggests the necessity of a longer period of incubation.

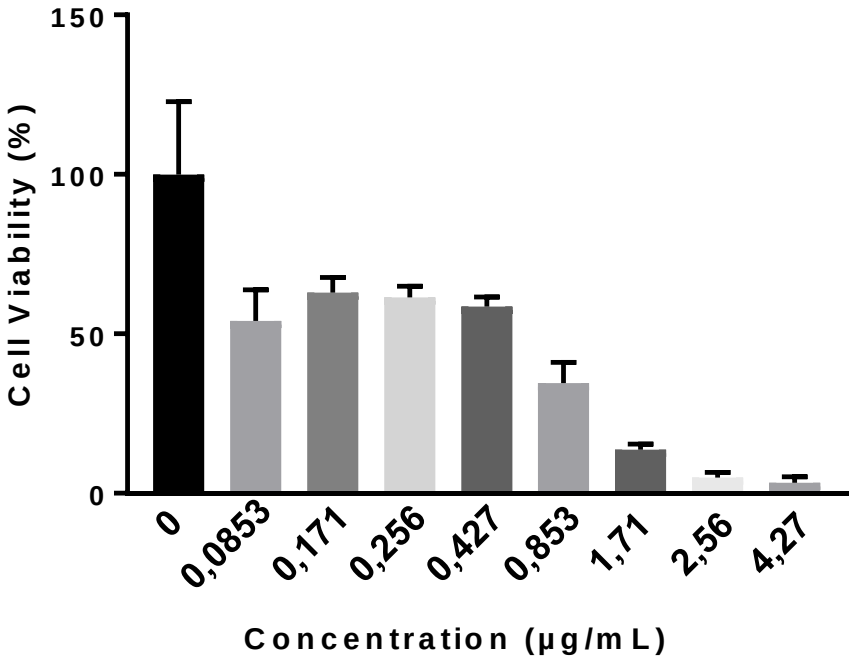

Figure 14 - MDA-MB-231 cell viability assessed by MTT assay as a function of the different concentrations of free PAC tested (0 µg/mL; 0,0853 µg/mL, 0,171 µg/mL; 0,256 µg/mL; 0,427 µg/mL; 0,853 µg/mL; 1,71 µg/mL; 2,56 µg/mL and 4,27 µg/mL). Values represent mean ± SD.

In addition, this assay offered useful information about which concentrations to evaluate with flow cytometry analysis.

3.5.2 Flow Cytometry

In order to evaluate apoptosis induced by the nanoformulations, MDA-MB-231 cells were stained by annexin V and 7-AAD and analysed by flow cytometry. Annexin V binds to phosphatidylserine (PS) which translocates to the extracellular membrane upon initiation of apoptosis. 7-AAD has high affinity to the DNA, thus label dead cells. In early stages of apoptosis only annexin V is detected, late stages of apoptosis with loss of membrane integrity allows staining by 7-AAD as well. Viable cells show no staining and cells stained solely by 7-AAD are necrotic[173]. Cells were treated under different conditions, empty or void NLCs, SPION-NLCs, SPION-NLCs-PAC, free PAC and free SPIONs for 24 hours and 48 hours.

Figure 15 shows that after 24 hours of incubation the percentage of apoptotic cells increases with higher concentrations of SPION-NLCs-PAC and free PAC, in relation to untreated cells. In comparison with cells incubated with NLCs and SPION-NLCs, it is also noticeable that there is a greater number of apoptotic cells when MDA-MB-231 cells are incubated with SPION-NLCs-PAC (about 5% at a concentration of 10 mg/mL in lipid). Therefore, the drug seems to be the main reason the cells are becoming apoptotic. Overall, as anticipated, free PAC seems to induce apoptosis at a higher extent than any of the other conditions (about 12% of apoptotic cells at the higher tested concentration of 0,013 mg/mL).

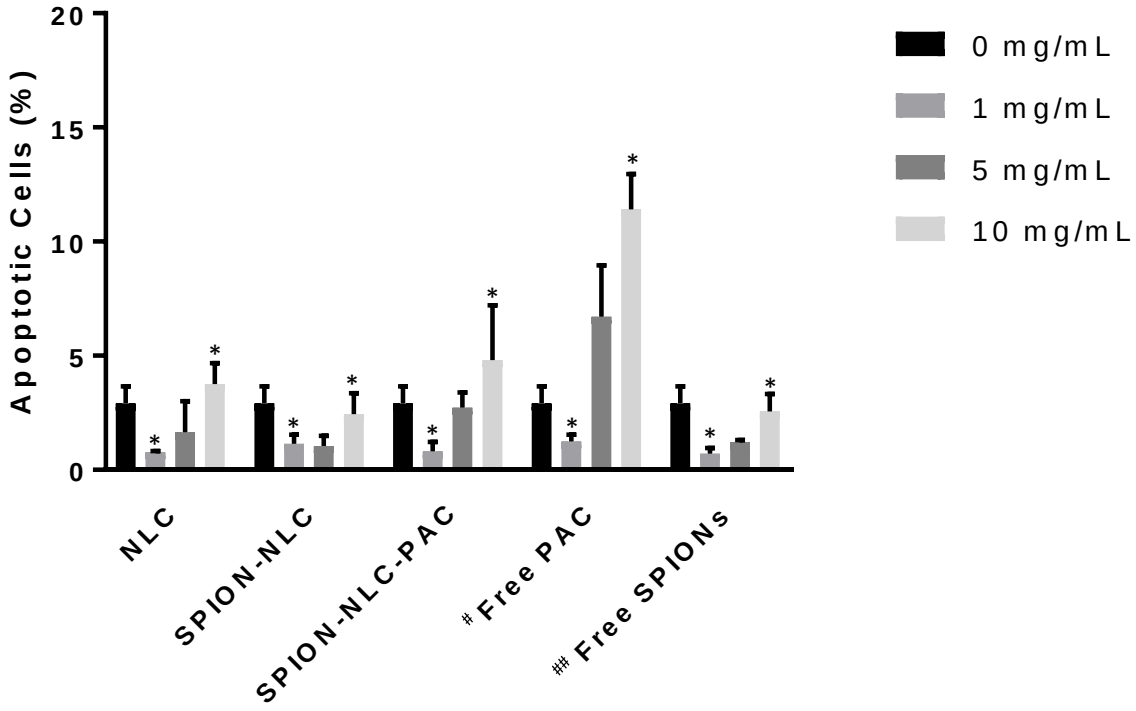

Figure 15 - Apoptotic MDA-MB-231 cells assessed by flow cytometry, after incubation for 24 hours with NLC, SPION-NLC, SPION-NLC-PAC, free PAC and free SPIONs (0 mg/mL, 1 mg/mL, 5 mg/mL and 10 mg/mL). Values represent mean ± SD. *P<0.05 relatively to the control. **Free PAC concentrations tested were the corresponding ones to the encapsulated PAC concentrations in NLC+SPION+PAC at 1, 5 and 10 mg/mL of NPs (0,13 mg/mL, 0,07 mg/mL and 0,013 mg/mL). * Free SPIONs concentrations tested were the corresponding ones to the encapsulated SPIONs in both SPION-NLC and SPION-NLC-PAC at 1, 5 and 10 mg/mL of NPs (,5 mg/mL, 0,2 mg/mL and 0,05 mg/mL).**

After 48 hours of incubation (**Figure 16**), SPION-NLC-PAC remains the formulation that induces higher apoptotic events for MDA-MB-231 cells (about 8% of apoptotic cells at a 10 mg/mL concentration). It could also be emphasized that with longer of incubation times more apoptosis is being induced by the NPs. Free SPIONs seem to have a higher effect on MDA-MB-231 apoptosis after 48 hours of incubation than detected after only 24 hours, displaying about 13% of apoptotic cells at the higher tested concentration. However, free PAC seems to have little to no effect on cell apoptosis, which could have been a problem with the assay, so another one should be performed.

This assay has shown promising results, though further investigation is clearly needed. An incubation period of 72h, as well as higher concentrations should be

evaluated in order to better assess the apoptotic mechanism of the SPION-NLC-PAC nanoformulation in MDA-MB-231 cells.

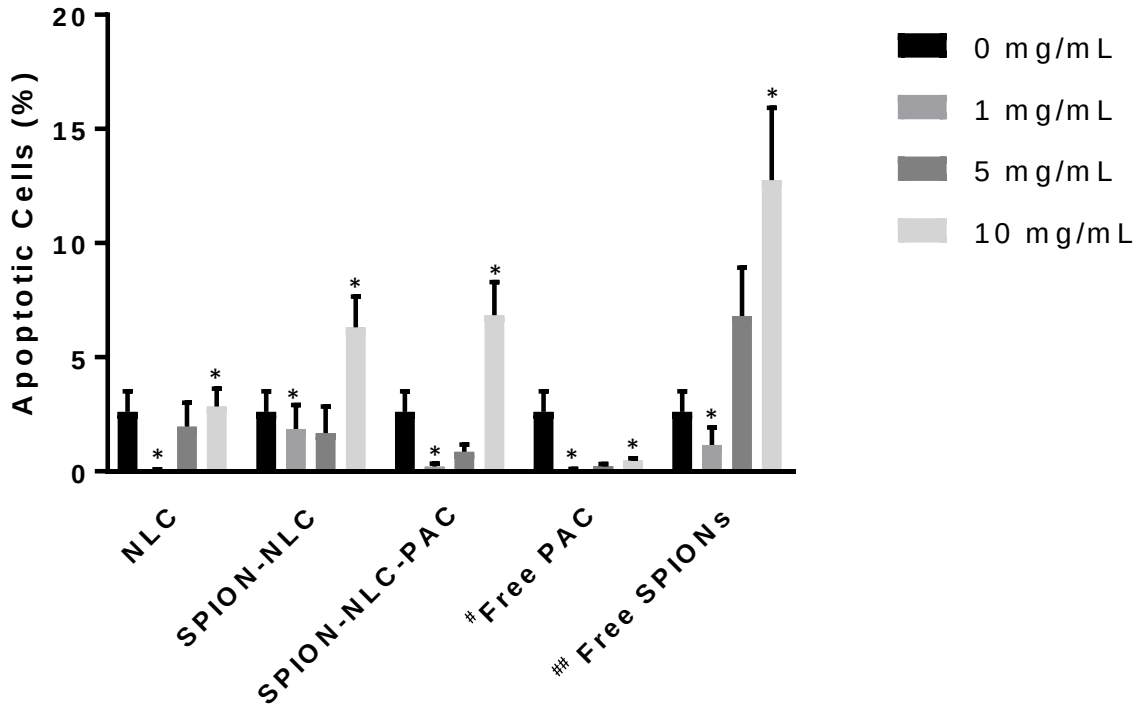

Figure 16 - Apoptotic MDA-MB-231 cells assessed by flow cytometry after incubation for 48 hours with NLC, SPION-NLC, SPION-NLC-PAC, free PAC and free SPIONs (0 mg/mL, 1 mg/mL, 5 mg/mL and 10 mg/mL). Values represent mean ± SD. *P<0.05 relatively to the control. # Free PAC concentrations tested were the corresponding ones to the encapsulated PAC concentrations in NLC+SPION+PAC at 1, 5 and 10 mg/mL of NPs (0,13 mg/mL, 0,07 mg/mL and 0,013 mg/mL). ## Free SPIONs concentrations tested were the corresponding ones to the encapsulated SPIONs in both SPION-NLC and SPION-NLC-PAC at 1, 5 and 10 mg/mL of NPs (0,5 mg/mL, 0,2 mg/mL and 0,05 mg/mL).

3.6 *In Vitro* Cell Uptake

When NLCs are intended for breast cancer therapy it is imperative to assess their uptake by breast cancer cells. Therefore, the uptake of SPION-NLC and SPIONs alone were evaluated. **Figure 17** shows that cell internalization of both SPION-NLC and free SPIONs occurs, being detected as early as 30 minutes after incubation. After 24 hours of incubation a 15% uptake of SPIONs can be observed, whereas only 7% of SPION-NLC are internalized after the same incubation time. In fact, SPIONs seem to be easily internalized when compared to the SPION-NLC formulation, probably due to their smaller size. These results suggest that the lipids decrease the uptake capacity of MDA-MB-231 cells. However, it should be taken into consideration that the lipids are fundamental in the delivery of the drug to breast cancer cells, the main objective of this project.

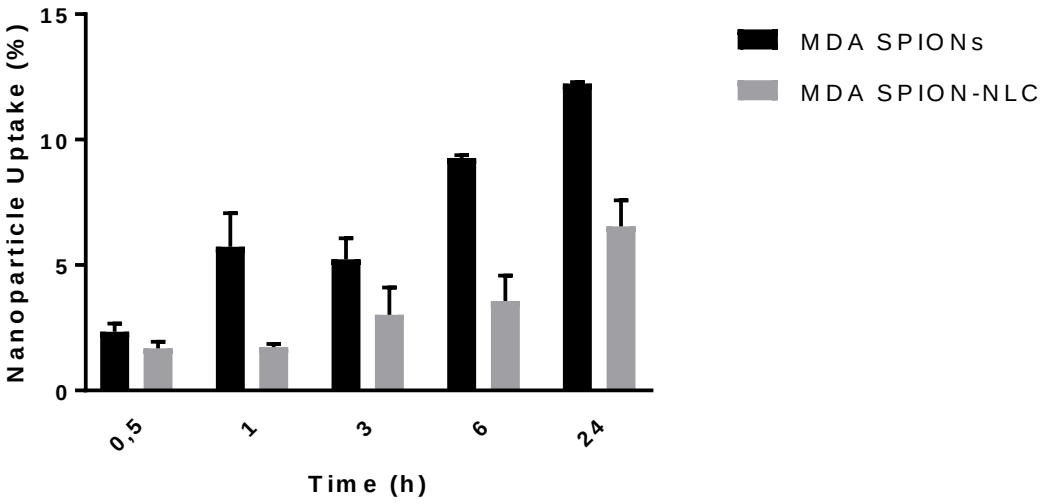

Figure 18 - Nanoparticle uptake by MDA-MB-231 cells up to 24 hours. Values represent mean ± SD (n=2)..

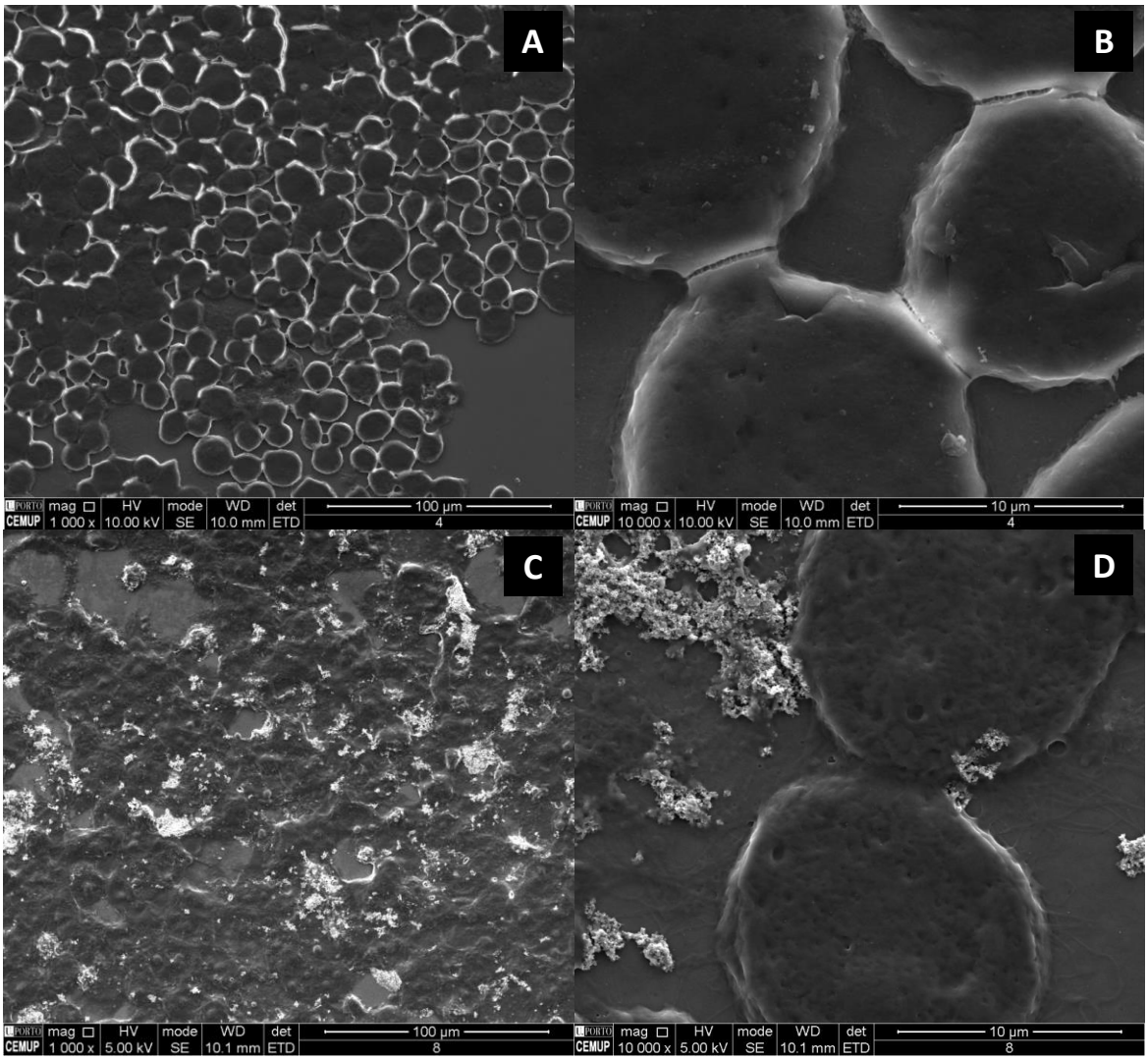

Figure 17 - SEM images of the NLCs suspensions. MDA-MB-231 cells, magnification 1000X (A) and 10000X (B). MDA-MB-231 cells after 24 hours incubation with free SPIONs, magnification 1000X (C) and 10000X (D).

3.7 Scanning Electron Microscopy (SEM)

SEM was used to evaluate the cells' and surface topography and composition. MDA-MB-231 cells treated with 0,5 mg/mL of free SPIONs, for 24 hours were observed. **Figures 18A** and **18B** show the morphology of untreated MDA-MB-231 cells. Brighter spots can be detected in **Figures 18C** and **18D** representing the SPIONs, as verified by the EDS spectra (**Figure 19Z1**), where a peak of iron is observed. Internalization of SPIONs by MDA-MB-231 cells can also be visualized in **Figure 20**, once again a peak of iron on the EDS spectra proves the particle inside the cell is indeed a SPION (**Figure 20Z3**). These results corroborate the results obtained with the uptake assay, suggesting that the MDA-MB-231 cells are able to internalize these SPIONs. In addition, cell morphology seems to not be affected by SPIONs uptake.

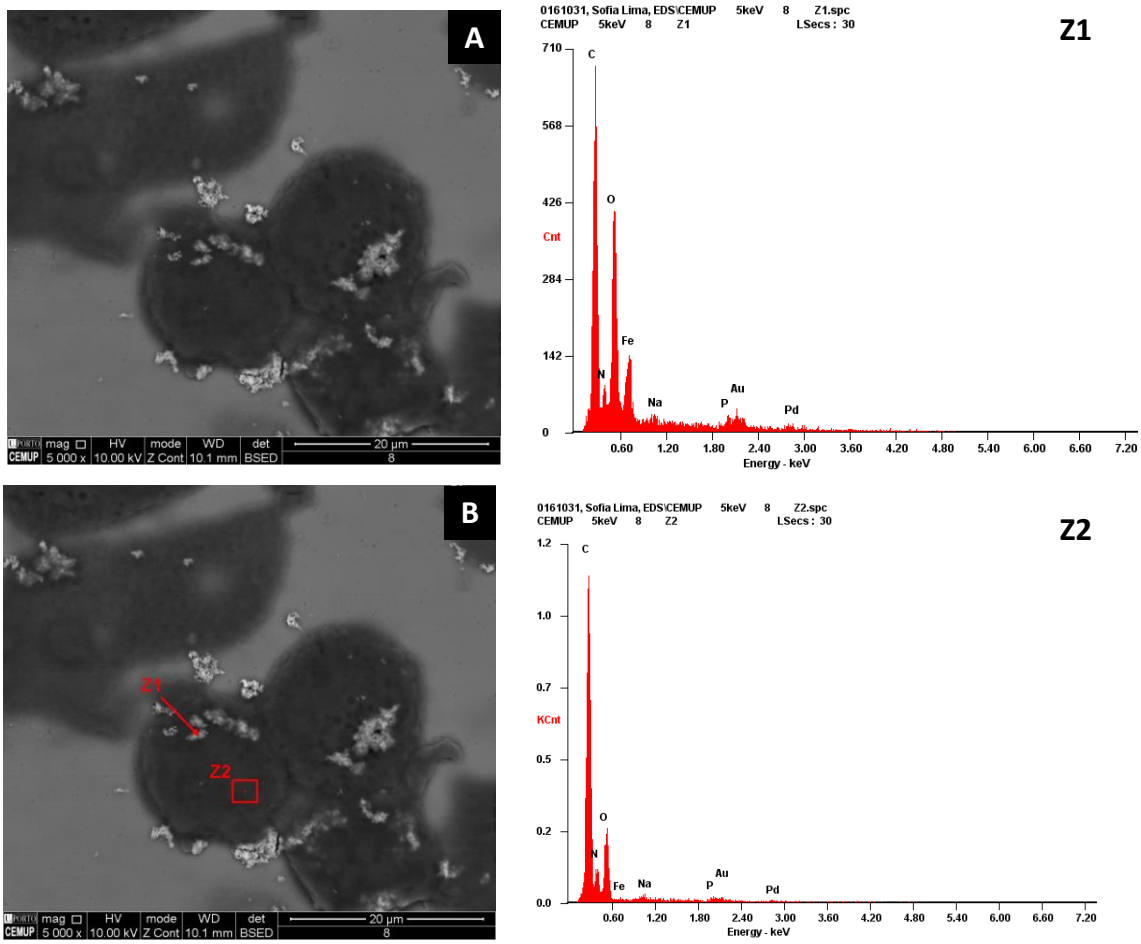

Figure 19 - SEM images (left) and EDS spectra (right) of MDA-MB-231 cells after 24 hours incubation with free SPIONs, magnification 5000X.

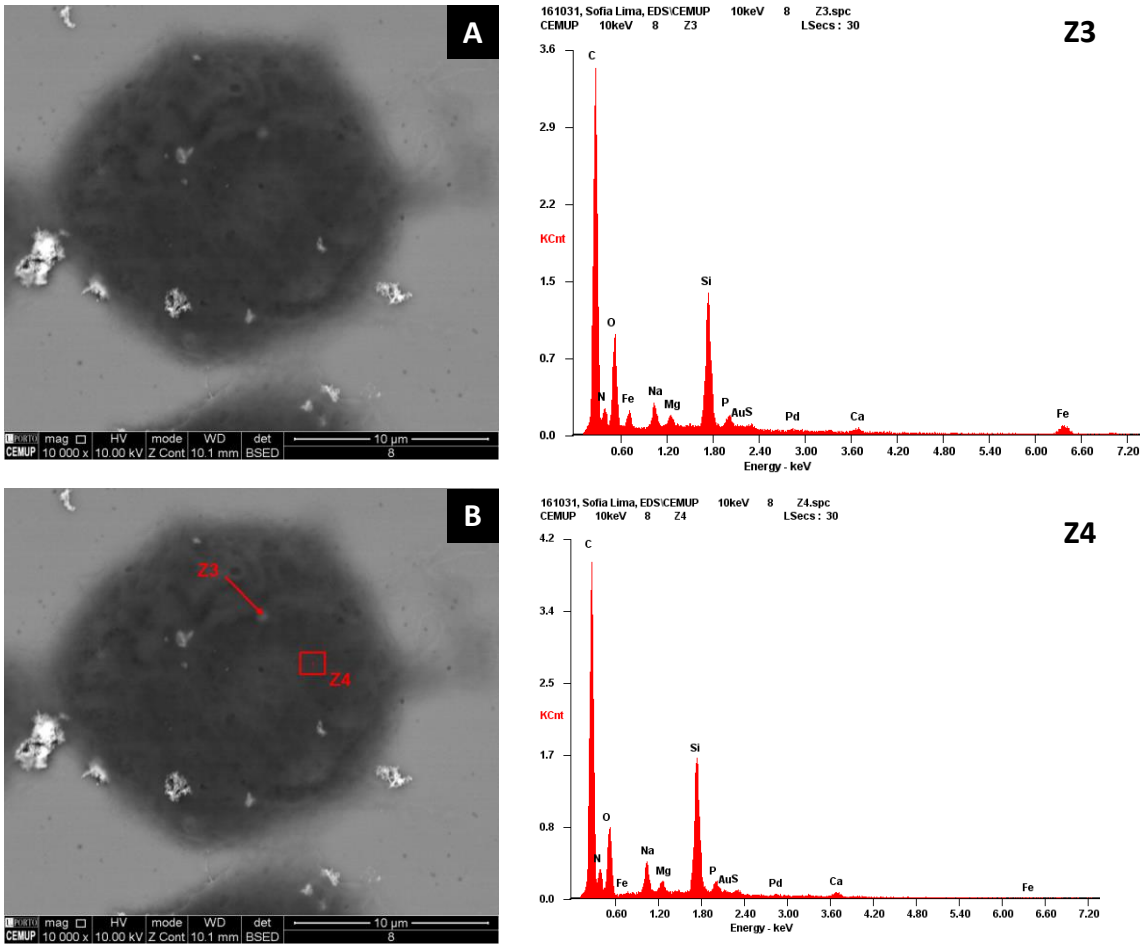

Figure 20 – SEM images (left) and EDS spectra (right) of MDA-MB-231 cells after 24 hours incubation with free SPIONs, magnification 5000X. An internalized SPION can be observed, marked by Z3.

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Chapter 4 – Conclusions

In the present work, PAC- and SPION-loaded NLC formulation was tested as a potential therapeutic agent for breast cancer therapy. These nanoformulations presented a size of 375 nm, a polydispersity index of 0,237, indicating monodispersity, a zeta potential superior to |30| mV, suggesting high stability, and a high encapsulation efficiency of 77%. In addition, NLCs stored at both 4ºC and 25ºC showed high stability, though storage at room temperature seemed to be the better choice. According to the literature, lyophilized particles are proven to maintain stability for greater periods of time, however lyophilization of the NPs was ineffective and the procedure must be optimized in order to obtain any definitive conclusions.

Internalization of the nanoparticles by MDA-MB-231 cells was proved in both the uptake assay and SEM. The free SPIONs were easily and more rapidly internalized by the cells, due to their smaller size. However, it is clear that the NLCs are indeed internalized by MDA-MB-231 cells. The cytotoxicity of the NLCs developed was assessed through MTT assays and flow cytometry. In the MTT assays cell death was recorded in the presence of the NLCs. Apoptosis induced by the NPs was assessed by flow cytometry, showing that the developed NLCs could be a valuable tool in breast cancer therapy.

In summary, it is expected that the nanoformulation diminishes noxious effects and allows controlled release and release on-demand due to hyperthermic capabilities of magnetic nanoparticles. Nevertheless, further studies are required in order to determine their exact effect on breast cancer cells.

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Chapter 5 – Future Work

For future work, stability studies of the NLCs suspensions stored at 4ºC and 25ºC are expected to continue in order to define the NLCs shelf-time. Release studies should also be performed in order to understand how the drug is release through time. It is also important to define a lyophilisation protocol that does not compromise the NLCs characteristics. These studies should be conducted at both 37ºC and 42ºC, since the first would mimic the nanoparticles response in the body and the second would mimic the tumoral environment. But before there is the necessity to optimize PAC's solubilty in sodium salycilate or another solution able to solubilize the compound. Functionalization of the nanoparticle surface would also be an interesting approach moving forward.

Further *in vitro* studies should be conducted. A wider range of concentrations and incubation times as well as different breast cancer cell lines should be evaluated. Ultimately, the efficacy of the designed nanosytem must be tested *in vivo* in healthy and disease animal model.

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